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IMMUNOLOGY AND CANCER

BY

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INTRODUCTORY REMARKS

By Albert S. Gordon

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The concept that the solution of the riddle of cancer is to be sought only in the cancerous cell has been strikingly refuted in recent years. It has become increasingly apparent that considerable insight into the cancer problem may be gained from a knowledge of the growth processes in the normal cell and from an analysis of those naturally occurring endogenous factors that enhance or inhibit normal growth. In fact, it has been the opinion of an insistent but well-grounded group of investigators that the basic etiological mechanisms in the production of cancer will not be known until we uncover the mechanisms for normal growth.

In this connection, our understanding of growth processes in the living cell has been aided by new stores of basic information constantly arising from the unlimited fountainhead of the morphologic, physiological, and biochemical sciences; this information is continually being applied to the problems of malignant growth. Thus we have noted the profits to be reaped from applying basic information derived from morphogenesis, cytochemistry, and cellular physiology to the cancer problem. We are now aware of the value of an understanding of the concepts of genetics for interpreting abnormal growth and its inheritance. The present monograph instructs us as to the value of yet another biological division; namely, immunology and its techniques, in both the comprehension and the combating of the cancer process. One is faced with the increasing realization that the investigator of cancer must bring to bear weapons derived from a variety of basic biological avenues in his attack on the problems involved.

The success which has attended the application of the basic disciplines of biology to cancer underscores a point that already has been emphasized; namely, that research in all basic fields of biology must continue unabated and unhampered if we are to continue to make successful inroads in the intriguing problem of normal growth and its equally interesting, but more vexing counterpart, malignant growth.

PREFACE TO IMMUNOLOGY AND CANCER

By Jerome T. Syverton

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Cancer has been the subject of intensive laboratory research for perhaps fifty years. Valuable advances have been made, but Nature yields her secrets reluctantly. It is not surprising that new knowledge of antigens, antibodies, and the mechanisms of immunity has stimulated concurrent and recurrent interest in cancer research. Unfortunately, this conjunction of disciplines has not been easy to effect. The truth is that both immunology and cancer research are advanced fields that are full of pitfalls for the uninitiated. The cancer biologist who wishes to make good use of immunological techniques must in many instances become both a competent biochemist and an experienced serologist, because the immunological methods necessary for the detection and measurement of the quantitative differences inherent in cellular systems were mostly devised for more rigidly defined reagents. The quantitative relations of simple antigen-antibody systems are sufficiently different, but when the complexity of myriad tissue antigens is compounded with the multifactor reactions of complement and the interactivities of serum, proteins, and isoantibodies, even the experienced immunologist may feel overwhelmed. The complement-fixation test continues to be a primary tool for the study of cancer immunology. If the difficulties of complicated serologic analysis are to be circumvented, one must still cope with the intricacies of protein separation and purification, with attendant dangers of the denaturation of antigen. Equally disconcerting is the finding that the basic mechanisms of tumor immunity are not limited or even dominated by the familiar classic antibody. Fortunately, the mixed breed of oncologist-immunologist is increasing, as is demonstrated by the number of imaginative approaches to immunological cell research recorded in this monograph. For those of us whose interest in tumor immunology outweighs our knowledge of immunochemistry, the work of the experts whose contributions appear here will help us to appreciate the principles, applications, and pitfalls of immunology in cancer research.

As we all know only too well, cancer is a most, even *the* most, demanding and frustrating field of biological research. The way in which solutions to basic problems of cancer have blandly and consistently eluded determined investigators for half a century leads one to suspect that our approach may have been incorrect. Again and again, in the history of cancer research, efforts have been made to reorient that approach. In the early years the general biologists, who had contributed so materially to such other fields as bacteriology and virology, attacked the cancer problem with equal vigor. Then came the geneticists with their very notable development of the inbred mouse. The biochemist and immunochemist have in turn taken up the battle. All of these groups singly have contributed tremendously to basic knowledge, and yet their work thus far has not been sufficient to achieve a solution. Years

ago it was suggested that the problem was too great for individual workers, and that research teams of associated biochemists, immunologists, geneticists, radiologists, and others, working together on directed and coordinated programs, would provide the answer. These teams also have made important advances, but cancer is still an enigma. The conference on which this monograph is based was convened as a congress of experts, in a sense: first, experts in methodology, but not necessarily cancer researchers; next, experts in the application of many of the methods to fields related to fundamental aspects of cancer biology; and finally, those who are actively engaged in cancer research. Possibly this is a potentially more rewarding approach to the prosecution of cancer research than the concept of research teams, or even of cancer biologists fully trained in *all* fields (virology, immunology, bacteriology, genetics, cell culture, and cancer biology). The latter type of investigator perhaps is ideally suited to cancer research, but it takes half a lifetime to train such a man. We are trying to do this at the University of Minnesota, and our achievement is slight. For those of us interested in cancer research, the synthetic approach represented in this publication indicates the uses and limitations of all sorts of advanced powerful techniques and, in turn, illustrates by examples problems in which these techniques are applicable. The latter are excellently summarized by H. B. Andervont, an expert in cancer research, in his interpretation of the direction and extent of progress in this field. When we organized the conference which has resulted in the publication of these papers we hoped that the synthetic approach would provide new ideas and concepts with warning of dangers involved: instead of a small synthetic research team operating under the restrictions of guided research, there was assembled a large international group for the exchange of information.

For many years the need for information concerning the basic principles of immunological techniques has been recognized. It is hoped that this monograph will serve to indicate the dangers to be avoided and the paths to be followed in achieving an understanding of the problems involved.

Part 1. Analysis of Cells

PHYSICAL METHODS FOR THE ANALYSIS OF CELLS*

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Introduction

A major and productive sphere of recent and contemporary research is that concerned with the isolation and analysis of "particulate" elements of cell derivation. Interest in and study of these materials, as distinguished from cellular substances classed as "molecular" in the accepted sense of the definition, have progressed in parallel with the development, principally, of physical equipment and procedures that provide not only access to the particles but, in addition, methods for examining and characterizing them critically. Although this has resulted in considerable success in some instances, there remain numerous unresolved problems in the general field, as well as outstanding omissions in the systematic use of the principles and procedures of well-recognized applicability. Fundamentally, the scheme of approach, as well illustrated by that devised and rigorously tested in investigations of viral agents, is relatively simple. This consists in (1) isolation of the particles of interest; (2) recovery (purification) of the particles in a given preparation that are homogeneous with respect to particle kind; (3) identification of the particles; and (4) analysis and characterization of the particles for the physical, chemical, or biological properties desired. Experience has revealed the shortcomings of the methods individually, but it has emphasized their productivity in correlated and complementary sequences. It is the purpose of this paper to reiterate the basic principles^{3, 5, 11, 30-32, 34, 37, 48, 49} involved in the physical isolation and analysis of particles from cells, with particular attention to work with viruses.

It is well to recognize at the outset that the predominant obstacles encountered in these studies reside in the problems of isolation and purification, especially the latter. That this should be the case even after more than twenty years of persistent application becomes more challenging with time. All of the observations made under adequate conditions have shown that the viral agents are well-defined physical entities, and that it ought not be too troublesome to devise a scheme of separation that would be useful for all of them, with only minor modifications to fit the individual instance. Such has not been the case, however. From the studies already made it is clear, as might have been suspected, that the impediments constitute a broad spectrum defined by extreme variations in physical and biological factors. In consequence, as it has proved, virus purification is usually a matter of art and

* The work reported in this paper was supported in part by a research grant to Duke University from the National Cancer Institute, Public Health Service, Bethesda, Md., in part by a grant from the American Cancer Society, Inc., New York, N. Y., awarded on the recommendation of the Committee on Growth of the National Research Council, Washington, D. C., and in part by the Dorothy Beard Research Fund, Duke University.

not of science, and a persistent trial-and-error approach may be more likely to succeed than one guided by purely theoretical considerations.

In contrast, identification, characterization, and analysis are subject to well-known disciplines established by intensive investigations, and the possibilities have by no means been exhausted. Strangely enough, it is in this field that the most frequent shortcomings are seen. These are related at times to evasion of disciplines or to their application to unsuitable materials. As was foreseen, much of the interest in the more tedious aspects of particle isolation and analysis has been diverted by the simpler and undoubtedly informative processes of electron microscopy. While much can be learned quickly with the instrument, it is nevertheless clear that the results obtained with it can never replace, and all too often may obscure, the need for the critical fundamental analyses that are dependent on access to homogenous materials.

Many physical methods have been applied to the study of virus particles, but those of principal usefulness are (1) centrifugation, first, for the qualitative fractionation of the source material, followed or accompanied by analytical centrifugation for the quantitative characterization of the significant preparations with respect to their properties, such as particle size, density, and shape, that govern the rate and manner of sedimentation; (2) electron microscopy, which is essential for guiding the development of the fractionation sequence, in morphologic definition of the product and, more recently, in the examination of virus in ultrathin sections; and (3) electrophoresis, for both fractionation and characterization. Ultrafiltration,²³ which formerly was useful for the determination of the approximate sizes of viruses, should probably receive greater attention than it now enjoys. All of these methods can be employed with profit with practically any virus, but it is under only unusual circumstances that the individual procedure is as productive as might be expected from theoretical expectations. Applicability varies widely from one entity to another, and no one method provides adequate criteria of the characteristics of the material or of the status of the preparations examined. It is the combination of all of these methods, together with ancillary procedures, which provides the basis critical for valid judgments.

Fractionation

Quantity centrifugation. Access to biologically important particles of cellular origin has been provided almost entirely by the development of centrifuges that afford high gravitational fields. Beginning with the oil turbine and, later, the air-driven machines, there have been developed the electrically powered and refrigerated instruments that are standard equipment in practically all well-furnished laboratories. All have been employed for the isolation and study not only of viruses, but of particles derived from normal cells.

The value of the centrifuge resides mainly in the provision of an efficient and relatively innocuous means for particle concentration. Its capacity as a procedure for fractionation (that is, the separation of different kinds of particles) is decidedly limited. It is a common misconception that particles are isolated

in homogenous preparations by differential centrifugation. Although so-called differential centrifugation could be effective under the requisite conditions, as Perrin²⁹ found in his work with suspensions of clay, the process is not generally applicable to biologically unstable particles that may be aggregated⁴¹ or damaged beyond usefulness by repeated sedimentation and packing, as are most of the animal viruses.

Centrifugation alone is effective in particle isolation and purification only under special conditions. These involve an overwhelming relative concentration of particles present in a medium containing no other particle population with sedimentation properties that approach those of the material of interest. The meaning of the term "concentration" of particles, as employed in the present sense, is concerned both with mass and particle number; the bearing of these factors on the processes of fractionation is illustrated in TABLE 1. The values given represent unit virus content, 1 mg. per gm. or ml. of the source material in relation to particle number. Mass varies essentially with the cube value of the radius and, consequently, it increases approximately eightfold with the doubling of the diameter of the particle. Thus, the content and potential yield of virus, other things being equal, increase very rapidly with increase in the size of the particle.

The significance of the values of TABLE 1, in terms of the possibility of virus isolation, can be illustrated by a few examples. Avian myeloblastosis virus, about 120 m μ in diameter, is present^{22, 28} in the plasma of chickens with this form of avian leukemia in numbers varying from about 2 trillion particles per ml. to levels too low for physical demonstration (less than about 1 billion per ml., as described below). Centrifugation of about 1 ml. of the best plasma results in a yield of approximately 1 mg. of hydrated virus.⁴⁰ In contrast, to obtain the same amount of virus from plasma containing only 1 billion particles would require a volume of at least 2 liters of plasma. Although this would not be too difficult to achieve, it would not be the end of the problem. Chicken plasma from both normal and diseased birds contains the equivalent of about 1 billion nonviral particles per ml. which sediment under the same conditions as the virus and cannot be separated from it by repeated cycles

TABLE 1
APPROXIMATE VOLUME OR WEIGHT OF SOURCE MATERIAL CONTAINING 1 MG. OF VIRUS IN
RELATION TO PARTICLE NUMBER AND SIZE, ASSUMING A PARTICLE DENSITY = 1

Particle number per ml. or gm.	Volume or weight of source material that contains 1 mg. of virus ml. or gm.			
	Particle diameter			
	30 m μ Poliovirus	60 m μ Papilloma virus	120 m μ Myelo- blastosis virus	240 m μ Vaccinia virus
100 million	1,059,750	132,469	16,558	2,070
1 billion	105,975	13,247	1,656	207
10 billion	10,600	1,325	166	21
100 billion	1,060	133	17	2.1
1 trillion	106	13	1.7	0.21

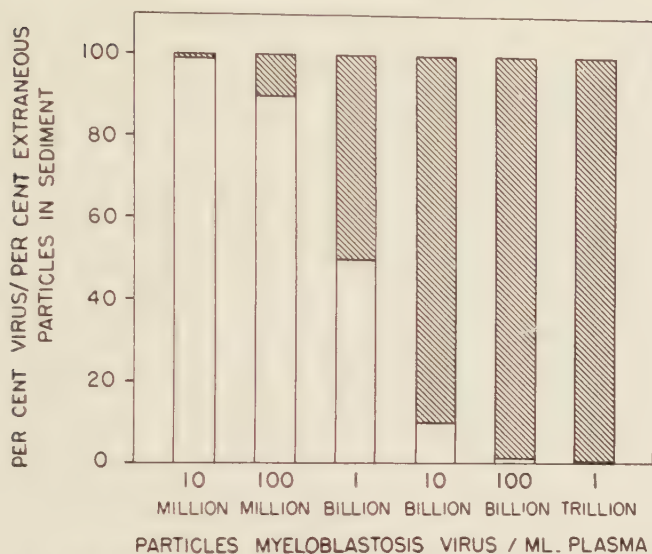


FIGURE 1. The ratio of virus (diagonal shading) to extraneous material sedimented from the plasma of chickens with myeloblastosis that contains about 1 billion nonviral particles in relation to different concentrations of virus particles in such plasmas.

of low- and high-speed spinning. The character of the product in relation to plasma particle concentration is shown in FIGURE 1. If one centrifuges about 1656 ml. of plasma containing 1 billion myeloblastosis virus particles per ml., the product would be 2 mg. (1 mg. virus plus 1 mg. of extraneous particles) of material (TABLE 1) consisting of 50 per cent virus and 50 per cent extraneous material. With increase in the number of virus particles, the proportional amount of virus increases in the pellets until, at the level of 1 trillion particles per ml. of plasma, the ratio of virus to other particles is 99.9 per cent virus to 0.1 per cent extraneous material (FIGURE 1).

In addition to these factors concerned with relative virus concentration is the matter of absolute amount available. It happens that in myeloblastosis sufficient virus can be obtained for many kinds of analyses. This is accomplished by selection of small numbers of birds with high virus content by means of the activity of the virus²⁷ to dephosphorylate adenosine triphosphate. A problem of a different magnitude arises with erythroblastosis,^{29, 38} in which the concentration of the particles of about 100 to 120 $m\mu$ diameter varies as in myeloblastosis, but it reaches levels not greater than about 100 billion per ml. Furthermore, it is not practically feasible to select the best plasmas, since the agent cannot be measured by any known enzyme activity, and both particle counts and infectivity measurements²⁰ are too tedious and lengthy in point of time to permit ready application. As a result, fractionation must be made blindly on plasmas that average about 10 billion virus particles per ml. As seen in TABLE 1, a volume of 166 ml. of plasma would be expected to yield about 1 mg. of virus in a mixture (FIGURE 1) which contains no more

than 90 per cent virus. In consequence, analyses with this agent can be of only limited application. The magnified difficulties with particles of smaller size are revealed in the results of calculations (TABLE 1) with agents of 60 $m\mu$ diameter, the papilloma virus,⁵ and the poliovirus of about 35 $m\mu$ diameter.¹ This also illustrates the basis for the early success with large particles (about 240 $m\mu$ in diameter) such as those of vaccinia.⁴⁴

The foregoing considerations have pertained to circumstances in which virus is present in an essentially acellular fluid medium in the absence of high concentrations of particles of nonviral origin. Other agents that occur under analogous conditions are the bacteriophages, which reach levels of at least 10 billion particles per ml. in lysates of the host bacterium; influenza, mumps, and Newcastle disease viruses reach high concentrations in the chorioallantoic fluid of chick embryos, and others, such as the poliovirus, in tissue culture. In all of these cases the agents occur in a relatively pure state by virtue of the processes involved in virus multiplication in association with specific host cells. Various preliminary treatments, such as filtration of the fluids with celite and bacterial filters, as well as autolysis, serve to produce suspensions that yield virus readily by simple concentration procedures.

In all of these instances the critical factor is that of virus mass in relation to the complexity or simplicity of the source material, and success has been experienced only with agents that are available either naturally under such circumstances or for which analogous conditions can be established by experimental means. It is likely, judging from the literature, that it was Ledingham²⁶ who first recognized and employed the principle of massive virus culture in host tissues of simple constitution. This was accomplished by inoculating vaccinia virus into broad areas of the epidermal cells of rabbit skin. Gentle scraping of the skin at the peak of the disease yielded pulpy preparations of high virus content in which the particles could be suspended by shaking briefly with glass beads. The virus could then be separated¹⁹ from the large masses of cell debris by centrifugation. It is noteworthy that grinding of the pulp ruined the purpose of the process by producing extraneous particles that could not be separated from the virus.

Successful isolation of virus from fleshy cellular sources is not often achieved except with the larger agents. Here, with the smaller particles, concentration in the tissue is especially important, and the proper conditions are encountered infrequently. This is well illustrated by attempts to purify the virus of equine encephalomyelitis cultured in chick embryos. In the embryo tissue the number of virus particles reaches levels of at least 100 billion per gm. of tissue,¹⁷ but there is present also a normal tissue component¹⁶ in approximately equal (judging from sedimentation diagrams) mass concentration. Although the virus is about 50 $m\mu$ diameter (sedimentation rate $s = 265$) and the normal component is 25 $m\mu$ ($s = 78$), no balanced sequence of high- and low-speed spinning compatible with recovery of the active virus sufficed to separate the components.¹⁷ A considerable degree of purification was accomplished, nevertheless, by allowing the ground chick embryo tissue suspended in saline solution to stand for a week in the refrigerator. Such treatment resulted, presumably, in the biological degradation and disappearance of the normal

component and left the virus as the predominant particulate material then separable from the molecular tissue proteins by simple sedimentation.

An unusual instance of purification of a virus by centrifugal fractionation is that of the agent of rabbit papillomatosis.⁶⁻¹⁵ The papilloma virus may occur in high concentration, recoverable in amounts of as much as 1 mg. (18 trillion particles) per mg. of warts, in the moist, denatured, and degraded (keratinized) host cells constituting the major portions of warty growths from cottontail rabbits. The virus is easily extracted in saline solution, but not the cell remnants, nearly all of which settle out of the suspension on standing, are easily thrown down by low-speed spinning or, preferably, removed by filtration with celite. If the warts are rich in virus, the pellets of the first sedimentation are essentially the pure agent. Resuspension easily disperses the virus, but the extraneous ("colloidal") material in this case only flakes up (unless pipetting is too vigorous) and can be eliminated by several cycles of alternate low- and high-speed spinning. It should be noted that the principle involved here is not the separation of particles by differential centrifugation, but one of the failure of the extraneous material to resuspend with the same facility as does the virus.

From these and analogous results it is apparent that the preparation of homogenous suspensions of biological particles has not been the result of finely and scientifically adjusted cycles of differential centrifugation, but of other physical factors; namely, those concerned with the interrelationships of the mass and characters of the materials involved. Thus, the act of purification must be effected, actually, by the host under natural conditions, or the interfering particles must be eliminated by treatment *in vitro* before the very first sedimentation. It has been entertaining, and frequently most frustrating, to be forced to relearn that the conditions operative will differ greatly from one entity to another.

The limitations of centrifugal fractionation as a procedure for purification have been well shown in repeated efforts to isolate virus particles from some cellular sources. This has been particularly the case with the tumor viruses. In this category fall the agents of the chicken sarcomas, avian lymphomatosis, mouse leukemia, and the mouse mammary carcinoma. The problems of the chicken sarcomas, as exemplified by the Rous tumor, have been considered very competently in recent reports.¹⁴⁻¹⁶ The reasons for the difficulties may be visualized by the results of a few simple calculations. If the virus content of the Rous tumor is of the order of 100 billion particles per gm., which is not unreasonable (see appendix to Bryan and Moloney¹⁶) and the diameter of the particles is about 100 m μ , it is evident that the agent is present to the extent of only 1 mg. per 20 gm. of tumor tissue. This is a ratio of 20,000 parts of tumor to 1 part of virus. Although much of the weight of the fleshy tumor is water and a watery solution of proteins that do not sediment with the virus, a relatively large proportion of cellular substance occurs as, or is converted in the process of grinding to, particles and fragments that are repeatedly spun down with the virus. Because of instability of the agent, drastic chemical separative procedures are not practicable prior to the first sedimentation, although there has been no dearth of attempts in this direction. The best

documented results have been those of Bryan and Moloney,¹⁶ whose final preparations have contained perhaps 1 per cent virus. This represents considerable relative purification, the result, mainly, of the use of growths of high virus content. Similar results have been seen in the laboratory at Duke University in studies of the cellular masses of avian lymphomatosis and the primitive cells¹⁷ of myeloblastosis obtained from the circulating blood. The unproductive results in these cases stimulated search for the agents of myeloblastosis and erythroblastosis in blood plasma which is simple in constitution and far more promising as the source material, although the particle content might be lower than that in the cells.

A possible means for circumventing many of the difficulties in the field of the fleshy virus-induced neoplasms is suggested by the results of studies of viruses grown in tissue culture. It has been shown that many agents (perhaps, under proper conditions, all of those that cause animal disease) can be cultured *in vitro* and find their way into the culture fluids in high concentration. Such has just been found⁹ to be the case in cultures of the primitive cells from the circulating blood in avian myeloblastosis. Occurrence of the agents in these fluids, even in relatively low concentration, may offer wide opportunity for exploitation.

A most important principle in the fractionation procedure under practical working conditions is that the process, by virtue of its objective, is wasteful. It is the purpose to eliminate undesirable components at a rate yielding the smallest ratio of nonspecific to specific material and not to the recovery of the largest amount of the specific particles. This requires the development of a sequence of centrifugal procedures in which the optimum proportion of the virus is sedimented and the remainder is discarded. The proportion to be recovered will depend on the constitution of the source material in relation to the amount and properties of the virus. In practice, the quantity of virus that can be recovered from suspension is not linearly related to the time of centrifugation. In experiments¹⁵ with the rabbit papilloma virus, for example, 50 per cent of the agent came down in 25 min. at $40,000 \times g$. In order to get one half of the residuum, 25 per cent of the whole, a total of 40 minutes was required, and all virus was down only after 70 min. With every increase in time, there was increase in amount in the pellet of particles smaller than the virus. As a general rule, the optimum sequence is one in which the rate and time of high-speed centrifugation should be adjusted to bring down approximately 65 to 75 per cent of the agent in a period of 45 to 60 min. Attempts to recover all virus, as practiced now and then, are useless, either for fractionation or for estimates of virus size.

A practical fractionation sequence is developed by simply trying various combinations of alternate high- and low-speed spinning and following the distribution of the agent in the different fractions by one means or another. In most instances, especially in the earlier work, this was done by measurement of infectivity, and selection of a good sequence required little experimentation, since the approximate sizes of most viruses are known from the older filtration experiments with gradacol membranes.²³ Knowledge gained by bioassay of infectivity may be augmented by other biological methods, particularly

those involving serologic tests, such as complement fixation or precipitation. These data are, in general, of limited value, since they indicate only where the virus is, with very little implication of quantity of virus or the state of the preparation with respect to the proportion of virus to other material. In many instances, bioassay is impractically cumbersome⁵ and, with viruses of long latent period, too time-consuming for effective work. The results, as indices of the amount of virus, likewise may be greatly misleading. Some agents are of very low virulence; that is, the median resistance of the host population, especially to tumor viruses,⁶ may be exceedingly high and, without knowledge of the relation of virus-particle number to infectious unit, there can be no judgment of virus amount.

Analytical centrifugation. In the years that preceded the existence of the electron microscope, the only really useful method for estimation of the physical state of the product and for direction of fractionation was that of analytical ultracentrifugation. Significant fractions could be spun in an optical system, and the rate and manner of sedimentation of the components could be followed in photographs of the descending boundary by several methods. The rate of sedimentation and the pattern of the diagram¹⁰ are dependent on the size, density, and shape of the particles. The procedure is highly effective with particles of uniform properties, in which case the descending boundary of a pure preparation is single and sharp. With the data obtained, the purification process (either that preliminary to centrifugation or, possibly, only that associated with fractional centrifugation) can be juggled until the product gives only a single sharp boundary indicative of a preparation with homogeneity in respect to properties of sedimentation. Analytical ultracentrifugation can be performed with relative rapidity and may be critically useful in guiding the process. A decided limitation is encountered, however, with many agents that vary greatly in uniformity of individual particle size and in other properties. In this case the boundary is diffuse, even with a homogeneous material, and there is no way of knowing whether the boundary is due to one or more than one population of particles.

Electrophoresis. Further check can be made by means of electrophoresis in the Tiselius apparatus. Here again, there is a severe limitation related to the quantity of virus required for a sufficient number of tests at significant *pH* levels. The phenomenon of migration in an electrical field provides a criterion of particle kind and properties wholly distinct from that afforded by other procedures. The rate and direction of movement are dependent on the magnitude and kind of electrical charge, presumably at the surface, and are independent of the size and shape of the particles. A population of particles homogeneous with respect to charge will migrate with a single sharp boundary. Thus, particles of the same kind, which may yield a very diffuse sedimentation pattern may nevertheless move at the same rate in an electrical field and yield a sharp boundary. Because of the various difficulties, electrophoresis is usually applied only after the more simple techniques have been well exploited.

Electron microscopy. Bioassay and analytical ultracentrifugation have been largely supplanted by electron microscopy and by procedures applicable

in combination with it. In almost all cases in which viruses exist in sufficient concentration and under conditions conducive to effective experimentation, electron micrographs will disclose almost immediately some evidence of the probable identity of the agent. This will be recognized either by characteristic morphology as the tailed bacteriophages, the rod-shaped plant agents, and the larger animal viruses, or by the appearance of a population of particles of relatively uniform characteristics. After exploratory shifts in the fractionation routine designed to increase the concentration of the presumably specific population, biological methods can be applied to confirm the electron micrographic findings and to provide the basis for further guidance by electron microscopy.

Identification and Quantitation

Despite the extensive advances already made in studies on viruses, the potentialities of physical methods for broadening and intensifying the field have been exploited only meagerly. Areas of increasing interest are those concerned with virus identification and quantitative correlation of the physical entities with chemical and, particularly, biological manifestations. Analytical ultracentrifugation and electrophoresis are methods that are dependent on relatively large amounts of virus in preparations of high homogeneity with respect to particle kind. Unfortunately, the purification of virus is always an unwieldy process and, furthermore, it is not always practical to carry it to a fruitful conclusion.

Particle counting. Other procedures, however, have been devised for rapid and quantitative experiments directly with crude or relatively crude materials and with those not easily susceptible to analytical ultracentrifugation or the standard moving-boundary technique of Tiselius. These are based on enumeration of virus particles^{27, 28} by electron microscopy and the correlation of particle number with other manifestations of the agents. For this there are two serviceable methods, each with its own advantages.

One process, developed by Backus and Williams,² consists in spraying onto a collodion membrane tiny droplets of a virus suspension containing a known concentration of easily recognizable index particles, usually polystyrene latex spheres. This has been employed²⁸ in the correlation of particles of bacteriophage and other viruses with infectious activity. Extraordinary usefulness was observed in development of the combined chemical and physical procedures that are efficacious in the purification of the poliovirus and the identification of the agent.¹ The procedure can be employed on some crude materials as well as on purified preparations, both in estimates of virus mass and in correlation of mass or particle number with various activities. Elegant results can be obtained with preparations that contain virus particles in numbers of about 2 billion or more per ml. This number, in terms of weight (density of the virus = 1.3) of the Lansing strain of poliovirus, for example, represents about 0.00003 mg. of virus, an amount present in 1 gm. of diseased brain from the cotton rat. The magnificent benefit of the technique is illustrated by the estimate that 33,000 gm. of brain would have been required to

yield 1 mg. of virus which, even then, would have been inadequate for study by other methods.

Another method for counting virus particles is that devised by Sharp³⁶ and later modified³⁹ for particular purposes. In the initial procedure, virus was sedimented from a known volume onto a collodion surface, dried, and shadowed; direct counts were made on a measured area of the film. This procedure was serviceable with viruses of characteristic morphology, such as the agent of influenza, in unpurified or purified preparations, and was applicable to suspensions containing particles in concentrations as low as about 10 to 100 million per ml. The process was useful with preparations containing physiological concentrations of salt or with those from which salt could be removed without damage to the virus.

Modification was necessary, however, for studies on the agents of avian leukemia, which are greatly distorted⁴² in the presence of drying salt. The difficulties were eliminated³⁹ by sedimenting the virus onto an agar block into which both salt and water diffused together. Films were obtained for direct counts in the electron microscope by fixation of the particles with osmic acid, by preparation of a pseudoreplica from the agar surface with collodion, and then by shadowing. Distortion was avoided with this technique and studies could be made of the particles sedimented directly from the diluted plasma of diseased birds.

Although virus particle counts by presently known methods are troublesome to obtain, they provide the most rapid and efficient means for estimation of virus amount and correlation of physical mass with the various properties of the agents. They are applicable to material of viral content far too low for study by other methods. An instance of recent experience is that of erythroblastosis virus³⁸ present in chicken plasma, as already mentioned, in numbers of about 10 billion per ml. Repeated studies can be made with large numbers of samples too small for other techniques and many of the more laborious, time-consuming, and less accurate procedures, including measurements of infectivity and serologic studies, can be circumvented.

Particle counting may constitute not only a major, but virtually indispensable, basis for viral identification and correlation with viral activity in the more difficult and complex circumstances, such as those encountered in investigations^{21, 22, 28, 43} on the viruses of myeloblastosis and erythroblastosis. In such cases, the procedure can be employed in association with analytical ultracentrifugation and electrophoresis or any other procedure designed to separate different kinds of particles. Such studies can be made on any material in which the concentration of particles is sufficiently great for electron microscopic identification. In work with ultracentrifugation, aliquots of the suspension are spun for different time intervals in an appropriate centrifugal field, and comparison is made of the number of particles in the various samples with the activities of interest. This can be done in narrow cylindrical centrifuge cells, and samples can be taken at a fixed level of the suspension or by use of a separation cell. The data provide also an approximation of the sedimentation rate of the particles. Similar types of suspensions can be employed for

electrophoretic fractionation. Employing the standard two-piece Tiselius cell, migration is instituted and allowed to proceed for an appropriate period determined by preliminary experiments. The parts of the cell then can be slipped, and each compartment sampled for particle count and comparative measurement of activity. Electrophoretic mobility can be calculated, likewise, from the distribution of particles within the various compartments.

The data obtained by all these procedures reveal (1) an accurate estimate of the number of particles as they occur in the source material or as they are derived in fractionated preparations; (2) the specific relationship of particles (that is, activity per particle) to other viral functions (infectivity, complement fixation, neutralization reaction, enzyme activity, precipitation, or antigenic behavior); (3) identification of particle with activity based on sedimentation rate (related to the size, density, and shape of the entity); and (4) identification of the particle and activity by the criterion of electrical charge. The total data, as obtained with the virus of myeloblastosis,^{21, 22, 28, 33} provide an unequivocal basis for judgment of identity or differences between the physical entity and activity. As in the work with the poliovirus, scarcely any other sequence of methods in the absence of particle counting could have permitted firm conclusions in the experiments with myeloblastosis. Measurements of infectivity⁶ with this virus are of relatively low accuracy, and the median population resistance of the host is too high (24 million particles per ID₅₀) for distinctive bioassay. The virus particles vary in size and give only diffuse boundaries in sedimentation patterns.¹⁰ Furthermore, quantitative electrophoresis effected by the moving-boundary method is not feasible because of the tendency of preparations to gel²¹ in the Tiselius cell at concentrations high enough for study. It would be scarcely feasible to attempt quantitative work with the virus of erythroblastosis by methods other than particle counting because of its low concentration in the plasma of affected birds.

Electron microscopy of specific precipitates. A decisive and frugal (with respect to material) means for the identification and correlation of virus particles involves use of the precipitin reaction at electron-microscopic levels. The potentialities of the procedure are well exemplified by its use in the identification of the virus of erythroblastosis.¹⁻¹³ With the low concentration of the agent in the plasma in this disease (the best source thus far recognized) there was little hope, without heroic measures, of identifying or purifying it by ordinary methods. The problem was resolved easily by concentration of the virus from 100 ml. of plasma to a volume of 1 ml. in which, after 2 cycles of sedimentation, the number of presumable virus particles was about 1.3 trillion. Because of the relatively large amount of extraneous amorphous particulate material (the equivalent of about 1 billion particles per ml. of plasma) in relation to the number of particles of uniform character (about 10 to 100 billion) the ratio of the former to the latter is always high (see FIGURE 1) in concentrates of this agent. After appropriate treatment of the obviously impure preparation with antierythroblastosis-immune serum from the chicken, the particles were sedimented on agar as in the usual counting technique. In the electron micrographs the particles specific to the disease were revealed in the aggregates produced by the action of the immune serum. Appreciation

of the result was enhanced by the absence of macroscopic evidence of precipitation. Thus, the technique revealed not only the physical nature of the particles precipitating, but provided unequivocal evidence that reaction actually occurred.

The value of the precipitative procedure as a means of correlation was shown again in experiments²¹ with the agent of myeloblastosis. Concentrates of this agent, as noted before, exert a strong adenosine triphosphatase activity and they also exhibit, by complement-fixation and neutralization tests with the sera of rabbits immunized with normal chicken tissue, the antigenic activity of normal chicken protein. The virus likewise appears to contain Forssman antigen, since it is neutralized by antibodies from rabbits injected with suspensions of guinea pig kidney. Treatment of the concentrates of the agent with antimyeloblastosis-immune serum from the chicken precipitates, quantitatively, the virus, as seen in the aggregates in electron micrographs and, at the same time,²¹ the enzyme and serologic activities. These findings fully corroborated the results obtained by methods involving analytical ultracentrifugation and electrophoresis, and they thus established the diverse activities and properties of the specific virus particles.

Electron Microscopy of Ultrathin Sections

An increasingly productive application of electron micrography is that of the investigation in ultrathin sections of the characteristics of particles as they occur within cells and after they have been shed or isolated from them. Developed in recent years in intensive studies of cellular structure,³⁴ the technique is being more frequently applied to cells diseased with viruses. Examinations of ultrathin sections afford the opportunity not only for identification of intracellular virus, but a growing insight into mechanisms that are involved in the processes of multiplication, including progression from the primitive to the mature forms of the agents. Unequivocally interpretable results have been obtained, especially with the larger viruses (molluscum contagiosum, vaccinia, and herpes) as well as the smaller adenoviruses, which are readily identifiable in preparations outside the cell.

A promising field for significant identification of the more obscure agents responsible for tumor induction is the study of tissue sections of the mouse mammary carcinoma,¹²⁻¹⁹ mouse leukemia,¹⁹ avian leukosis,¹⁹ and the related neoplasms^{7, 33} of the avian sarcoma group. In erythroblastosis of the chicken, intracellular particles have been observed¹⁹ that are of the same morphology as that of particles in concentrates of the agents of erythroblastosis and myeloblastosis as demonstrated by W. Bernhard and Françoise Haguénau of the Gustave-Roussy Cancer Research Institute, Villejuif, France, in collaborative studies with Duke University. The erythroblastosis virus occurs in reticular cells of the spleen as intramitochondrial elements. Similar to the viruses of erythroblastosis and myeloblastosis are the structures seen in cells of the Rous sarcoma²⁵ and the Murray-Begg tumor.³³ Particles that unquestionably appear to be the agent of the mouse mammary carcinoma have been found^{12, 19} repeatedly in the cells of the growth, and structures of great probable significance are present in cells in mouse leukemia.¹⁹

It is likely, as the results already suggest, that an outstanding contribution of ultrathin sectioning will be the clarification of obscure questions of virus occurrence and host-virus relationships in the problem of latency. This has been a difficult matter, particularly in the study of tumor viruses. In mouse mammary cancer and mouse leukemia especially, the identification of particles as virus outside the cells has not been effected, and in these growths, as well as in rabbit papillomatosis, in the avian sarcomas, and in the avian leukemias, which are known to be of viral origin, the agent may not be demonstrable by transmission experiments. A long-delayed manifestation of virus activity is an outstanding phenomenon in mouse mammary carcinoma and one strain of mouse leukemia. Still more confounding is the complete absence of outward virus effects in hosts of susceptible strains brought experimentally into contact with virus. Ultrathin sectioning provides the only means presently available for the search for specific agents in the tissues of the apparently undiseased host. That search by this means can be productive, though exceedingly tedious, has been illustrated by the demonstration of particles of significant appearance in tissues associated with the diseases of mouse mammary cancer and avian neoplasia. Since the techniques of ultrathin sectioning have been developed to the level of study of particle structure itself, it may be expected confidently that we shall learn a great deal about the occurrence and characteristics of the tumor viruses that may lie dormant without causing disease in the life span of the host.

A prospective application of ultrathin sectioning that is particularly stimulating to those interested in cancer is the systematic search for specific cellular structures in growths not presently known to be of viral etiology. Many studies of this sort have already been made. The results in some instances have been most suggestive. Outstanding in this respect are the cytoplasmic formations in cells of the Ehrlich mouse ascites tumor.⁵⁵ Dalton and Felix¹⁸ have reported the occurrence of particles in mouse tumors S37 and melanoma S91 that are similar in appearance to the presumptive virus in chicken tumors. Some of the cells of spontaneous hepatomas of mice contain viruslike particles,²⁴ and such particles have been seen in a chemically induced tumor of the chicken.³³ Obviously, these observations are subject to many interpretations with respect to the nature of the particles and their bearing on the occurrence of the growths. Although it may be found in time that such structures are either nonspecific agents carried along with the tumor or are of nonviral origin, it is undesirable to dismiss them without thorough attempts at identification. The experience in the past twenty years has certainly shown that the failure of cell-free transmission cannot be accepted as evidence that a given tumor is not of viral etiology.

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CHEMICAL METHODS FOR THE ANALYSIS OF CELL FUNCTION

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I should have been better pleased with the title of this paper, which I did not select, if I could have altered it in such a way as to include mention of physical as well as chemical methods for the analysis of cell function, for we cannot limit ourselves to chemical methods in such an analysis, since so much of our work depends on the use of physical methods in combination with them. The basic methodology employed by a great many biochemists today involves the physical separation of cell homogenates into fractions that are hopefully labeled nuclei, mitochondria, microsomes, and soluble fraction, although most of us are aware that the tissues with which we deal are mixtures of different types of cells, and that the fractions that we thus label are only operational at best. A great part of this paper draws heavily on the work of George Palade and Philip Siekevitz, whose work on the biochemistry of the microsome fraction seems to me to be one of the most significant recent developments in biochemistry, especially insofar as the purposes of this monograph are concerned. The fact that a chemist of the stature of Erwin Chargaff of Columbia University, New York, N. Y., is also working on this problem should give us added hope that useful studies on this fraction will be performed.

If we ask what are some of the modern methods of chemical analysis of cell function, we immediately come to the fact that combinations of methods with modern automatic instrumentation now provide us with opportunities for experimentation that could not have been imagined ten years ago. For example, I refer to the magic of the ion-exchange columns, whose usefulness was recognized and developed by W. Cohn.¹ This method, combined with the automatic collection of fractions and the automatic increase in the concentration of the eluting solutions² (FIGURE 1), opens up new horizons in the analysis of the metabolic pools that occur in tissues. In most cases, however, we are not content to know the concentrations of the various compounds, but we wish to know from what they are formed, to what they are converted, and how rapidly these processes are occurring. Here we find the isotopically labeled precursor a powerful tool that was developed just in time to be used in conjunction with the newer methods of compound separation. A recent paper³ from our laboratory shows the results of the combined use of these methods (FIGURE 2). These data are unintelligible as they stand, but when similar data were collected 2, 15, and 60 min. after rats were injected with P^{32} , and the specific activities of the 12 major nucleotides were calculated for each time point, it was found that all of the polyphosphate nucleotides were rapidly labeled at about the same rate. We conclude that adenosine triphosphate (ATP) is only one of a number of energy-transmitting nucleotides. I should devote more space to the acid-soluble fraction were it not for the fact that I believe that the reader is interested primarily in the acid-insoluble fraction



FIGURE 1. Apparatus for the automatic increase in concentration of eluent (gradient elution) and the automatic collection of fractions.² The pointer shows the top of the ion exchange column at the initial position of the sample. To the left are the reservoir and the mixer.

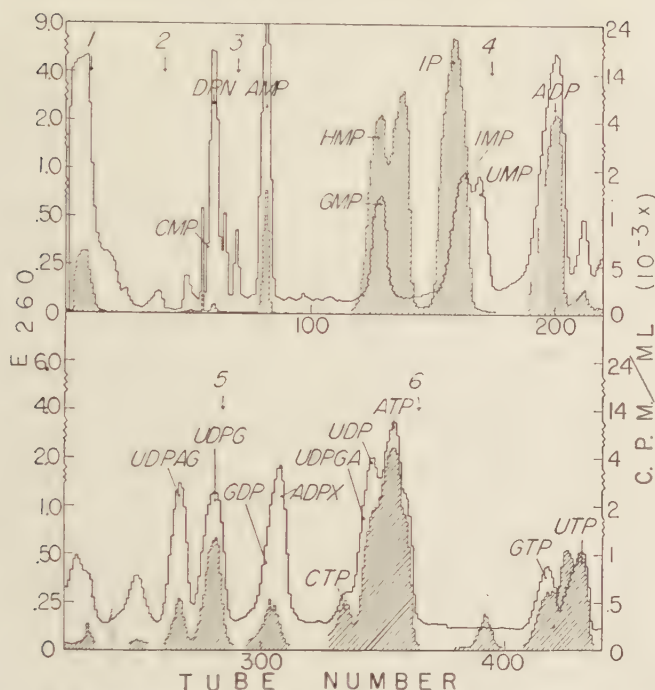


FIGURE 2. A chromatogram showing the distribution of acid-soluble nucleotides and radioactivity (cross-hatched) from 10.8 gm. of rat liver 15 min. after the injection of 100 μ curies of radioactive inorganic phosphate. The data are from Brumm, Potter, and Siekevitz,³ using the type of apparatus shown in FIGURE 1. Reproduced by permission from *The Journal of Biological Chemistry*.

of tissues. We study the acid-soluble fraction by means of chromatographic separations and isotopic techniques primarily in order to evaluate the function of the acid-insoluble macromolecules. What can we learn of the macromolecules themselves, their identity, their function, and their interrelationships? Here we find the common ground on which the final understanding of cell function must be achieved. Here we find an opportunity to combine cell fractionation with isotopic techniques and chromatographic separations. In considering the macromolecules we think of the nucleic acids and the proteins that combine to form the nucleoproteins and the other conjugated proteins. The function and synthesis of these macromolecules are closely interlocked in a feedback network that involves the acid-soluble nucleotides referred to earlier, but the functioning of this network will not be understood until the internal anatomy of the cell is comprehended. Meanwhile, we can try to understand the role of the various parts of the cell on a tentative basis. We can say little about lipoproteins at this time, but we know that they are involved in the structure of membranes, and that their functional role is in urgent need of more study. The synthesis and possible function of the nucleoproteins are currently receiving considerable attention because the metabolic function of these macromolecules seems to be within the range of present methods.

The Microsome Fraction

The discussion at this point can perhaps best be oriented in terms of the structures that can be visualized with the electron microscope and defined in terms of nucleoprotein content and function. We are concerned with the nucleus, which contains all of the deoxyribonucleic acid (DNA); with mitochondria, which contain the mechanisms for oxidative phosphorylation and numerous syntheses and a small amount of ribonucleic acid (RNA); and with the endoplasmic reticulum (Porter *et al.*, as cited by Palade¹), which consists of membranes in the form of hollow plates and tubules of lipoprotein plus what we shall refer to as "Palade particles,"⁴⁻⁷ containing RNA,^{6,7} all bathed in a solution of soluble proteins and small molecules. In all cases it appears that the nucleic acids are combined with proteins. Very few investigators have the background or interest necessary to relate these structures to the preparations that are studied by the biochemists who use homogenates and cell fractions, but Palade and Siekevitz have done so,^{6,7} as have several others, including Novikoff,⁸ who has recently reviewed the work in this field. From these studies it seems clear that what workers in this field in the past have called "microsomes" is a suspension of pinched-off membranes and tubes that have re-established some kind of a structural continuity, together with a substantial fraction of the Palade particles. The resulting rounded structures or "vesicles" behave as osmometers and swell in hypotonic solutions.⁶ These recent studies are of considerable importance in assessing the meaning of the former and contemporary studies performed by biochemists with the "microsome" fraction. It is clear that this fraction is an operational term, and that it is preferable, though not always convenient, to speak of the microsome *fraction* rather than of microsomes, as if they were particles of a specified size that actually occur in cells. This view does not deny the possible existence of additional small particles in this fraction. It appears that the Palade particles appear as such in cells, but that most of the "vesicles" with which they are associated are part of more elaborate structures. I believe the separate studies by Kuff *et al.* (reviewed by Novikoff⁹) are not incompatible with this view. In all further discussions we shall use the terms microsome fraction or microsomes to mean the combined constituents of membrane and RNA. The term Palade particles will be used in its strict sense. Under existing operational conditions, part of the Palade particles are thrown down into the microsome fraction, and part of them remain unattached to any membrane protein and remain in the supernatant (S_a) fraction with the soluble proteins.⁷ Continued high-speed centrifugation appears to increase the yield of the Palade particles in the pellet,⁷ but in our laboratory the supernatant fraction has never been obtained free from RNA, which may imply that this fraction still contains Palade particles. Moreover, it may be noted that in metabolic studies we have consistently observed that the specific activity of the RNA from the supernatant fraction is higher than that of the microsome fraction.

Subfractionation of the Microsome Fraction

I shall first consider the chemical properties of the microsome fraction, and later discuss the functional aspects that may be of interest to the reader. Palade and Siekevitz^{6, 7} have treated the microsome fraction with sodium deoxycholate, which appears to solubilize the membranous elements and to leave the Palade particles free in suspension.⁶ When these investigators re-centrifuged the suspension at $105,000 \times g$ for 2 hr. the Palade particles were in the pellet, while most of the protein, the hemochromogen, the phospholipid, and the DPN-cytochrome *c* reductase were no longer present, and were probably in the supernatant fraction, although the reductase apparently had been partially inactivated by the solubilization procedure. What is of greatest interest here is the question of whether the protein that remained in the Palade particles was intrinsically part of their structure. This question has been pursued further by Chargaff, Elson, and Shiguera,⁹ who studied the effect of deoxycholate over a wider range of concentrations. They found that above concentrations of about 0.5 per cent the ratio of protein to nucleic acid became constant and, indeed, seemed to level off at a whole number, which suggested a nucleoprotein structure in which there were 2 nucleotides per amino acid residue. From the results obtained in both laboratories, it would appear that the microsome fraction contains a large but variable fraction of its protein in the form of membranes that are not nucleoprotein in nature and are probably lipoproteins, plus a small fraction of protein in combination with RNA, possibly in a fixed ratio. Since the Palade particles appear to be attracted to the membrane proteins, it would be of interest to learn the nature of the forces of attraction and whether they are of physiological significance.

Function of RNA Proteins

In searching for the function of the Palade particles it must be admitted that most of my opinion comes by analogy from microbiological studies, notably by Spiegelman¹⁰ and by Gale,¹¹ which imply definite functions for RNA and a reciprocal synthetic function for RNA and protein.

Of very great interest is the report by Pardee and Prestidge¹² on the catalytic effect of amino acids in the synthesis of RNA in the absence of protein synthesis, and the suggestion that amino acid nucleotide derivatives might be intermediate in the synthesis of nucleoprotein on a nucleoprotein template. Similar data by Gros and Gros¹³ have appeared and seem closely related to the work of Hoagland,¹⁴ Hoagland, Keller, and Zamecnik,¹⁵ De Moss and Novelli,¹⁶ and of Davie, Konigsberger, and Lipmann.¹⁷ These studies have yet to be integrated with the finding by Ochoa¹⁸ and others that RNA can be synthesized by certain bacterial enzymes acting on nucleoside diphosphates with no hint as to any template or specificity of structural pattern. Possibly there are two categories of RNA, one for storage of information, and one for storage of nucleotides, each category being formed from different types of nucleotides or derivatives thereof, such as nucleoside diphosphates for the Ochoa type and amino acid nucleoside monophosphates for the other. In the case of information storage, the basic assumption is that the enzyme-forming system (EFS)

is a combination of RNA, protein, and inducer, the protein acting as an enzyme when not in the complex, and the inducer being either the true substrate or a configuration of atoms that can induce a protein configuration in the EFS that will be complementary to the substrate.¹⁰ These studies also assume more or less explicitly that the EFS is the amplification or production system for genetic information carried in the DNA.

In the case of animal tissues, present information is fragmentary, and it is usually interpreted in the hope that chance will favor the prepared mind, that is to say the mind prepared in part by the experience of the microbiologists. The problem has three aspects: (1) protein synthesis, (2) RNA synthesis, and (3) net increase in specific enzyme concentration. The last, of course, would require demonstration in order to provide evidence for the functioning of an enzyme-forming system.

There has been ample evidence that the microsome fraction and, indeed, the RNA therein, are concerned with protein synthesis. This line of evidence does not dispute the evidence for protein synthesis by RNA systems in the nucleus. It seems likely that protein synthesis occurs in both sites. In the microsome fraction, studies by Borsook,¹⁹ Allfrey, Daly and Mirsky,²⁰ Siekevitz,²¹ and Zamecnik and Keller²²⁻²³ all point to this fraction as an important site of protein synthesis.

Less evidence is available regarding RNA synthesis in the microsome fraction. However, studies by Herbert, Potter, and Hecht²⁴ strongly suggest that the microsome fraction is the limiting factor in the incorporation of labeled nucleotides into RNA, although no net synthesis has yet been demonstrated.

In discussing protein and RNA synthesis in the microsome fraction I have considered only the *in vitro* cell-free systems, and in this category there are no cases of net enzyme synthesis. However, it should be emphasized that knowledge of the management of the nucleotide pools is slowly increasing (Herbert and Potter²⁵) and, as knowledge increases on how to maintain the energy supply and how to control the supply of building blocks without unwittingly setting up normal blocking mechanisms (Yates and Pardee²⁶), it seems likely that we shall move into an era in which intracellular functions such as adaptive enzyme formation and virus reproduction will be brought about in cell-free preparations from mammalian tissues.

Meanwhile, a new combination of methods has been developed (Hecht, Brumm, and Potter²⁷) that seems to have many useful applications. Tissue slices were incubated for various periods of time, either with a radioactive precursor or shortly after the injection of a labeled precursor. After various periods of time *in vitro*, the slices were removed and homogenized, and centrifugal fractions were prepared. The nuclear RNA was found to increase its radioactivity without a lag period, while the various cytoplasmic fractions showed a delay in the attainment of their maximum rate. This method has also been combined with the use of agents that block nucleic acid synthesis in order to assist in evaluating the relative roles of nuclear and cytoplasmic RNA. In these experiments, as well as in cell-free systems,²⁸ the radioactivity

of the RNA in the supernatant fraction has always been much higher than that in the microsome fraction, which indicates that the unattached Palade particles may more actively synthesize RNA than do the attached particles, or that they may be more intimately related to nuclear activity.

Relation to Cancer Problem and Immunology

The foregoing remarks would have little relevance here if they could not be related to immunological techniques and to the cancer problem. To me, the most exciting thing about the ribonucleoprotein of the cytoplasmic fraction is that, according to Palade,^{5, 6} it appears to be particulate and to occur in uniformly sized packages about 100 to 150 Å. in diameter. If, indeed, these particles are (1) to be identified with the EFS of mammalian tissues, (2) to be the basis for the enzyme pattern found in cells, and (3) to be responsive to enzyme-inducing substances, their physical appearance of uniformity must be highly misleading. It seems more likely that there would be a special EFS for each *kind* of enzyme, while the *amount* of enzyme would depend upon the presence of inducers or substrates in addition to building blocks and energy available to each kind of particle. I should assume that some of the EFS are replaceable by virtue of DNA prototypes existing in the nucleus, while others are not backed up by DNA prototypes. I should also assume that each EFS or its DNA prototype forms proteins that are important in determining morphology and tissue-specific antigens. In 1950 Potter, Price, Miller, and Miller²⁹ published a diagram to suggest how the concentration of *particulate*

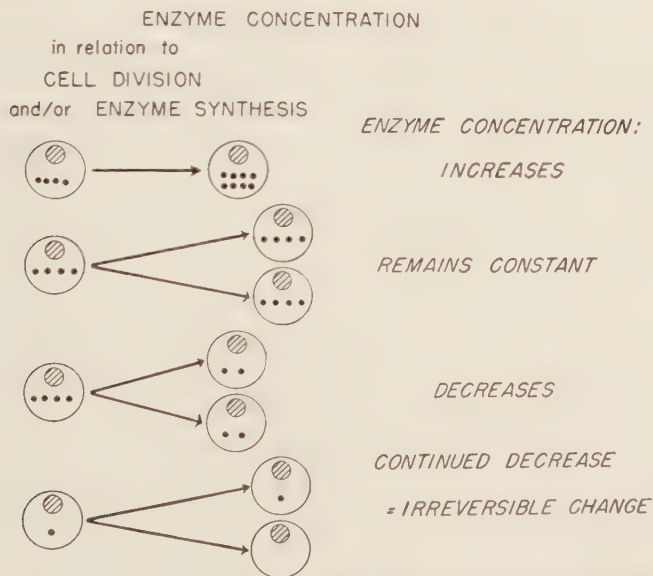


FIGURE 3. The significance of particulate distribution of enzymes, enzyme forming systems (EFS), and RNA in cells.²⁹ The properties of the cell line can be altered by variation of the rate of cytoplasmic particle duplication relative to the rate of cell reproduction. Reproduced by permission of *Cancer Research*.²⁹

enzyme systems might vary by increasing or decreasing at rates not proportional to the rate of cell division; this figure is reproduced here (FIGURE 3) because the same principles should apply to the EFS. The problem of differentiation may be looked upon as the relative gain of some EFS systems, as a loss in others, and as a complete deletion of others. My present view of the cancer problem is that normal cells are most likely to be converted to cancer cells by a *succession* of irreversible losses of EFS, each of which produces a catabolic enzyme, the action of which is competitive to a synthesis. According to this view, not all cancers should be expected to have the same enzyme pattern; if this is so, how can we expect to define the enzyme pattern of cancer tissue? I believe that the best answer to this is that a continual search for qualitative as well as quantitative differences in enzyme content, as well as for intracellular distribution, must be made in a wide variety of tumor and normal tissues in order to seek common denominators among tumors. Meanwhile intensive studies on single tumor types in comparison with homologous normal cells must be made.

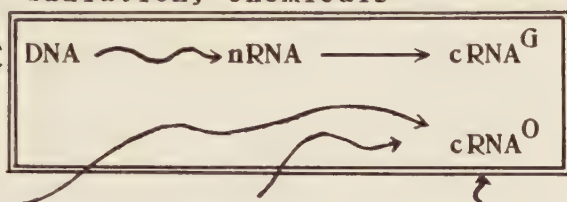
I believe that in the attack on the cancer problem the methods of immunology should be combined with cell-fractionation studies and the methods of enzyme assay. Thus far the separate experiments have been performed, but I do not believe they have been combined. The work of Eberhardt Weiler in Tübingen, Germany, seems to suggest a way in which immunology could supplement the studies by enzyme chemists. Weiler²⁰ showed that the microsome fraction of primary liver tumors contained nondetectable amounts of the so-called liver-specific antigen, while the precancerous livers of animals that were fed the carcinogen contained lower amounts of the antigen than did normal liver cells. His histological sections, employing fluorescent liver-specific antibodies, showed what appeared to be clones of liver cells with lowered amounts of the liver-specific antigen; in many ways, the work he reported seems to support the theory of carcinogenesis outlined above and illustrated in FIGURE 3. However, what must be done now is to learn whether the tissue-specific antibodies react with the RNA particles or with the membranous proteins, and whether a specific enzyme or group of enzymes is precipitated from the liver microsome fraction when it is treated with liver-specific antisera. Can tissue-specific sera be used to alter selectively the enzyme pattern of a tissue fraction? Presumably we should be able to recover detectable amounts of enzyme from the RNA-protein fractions if we can remove the RNA without denaturing the protein. Possibly this could be done in the presence of the substrate of the desired enzyme. What is the relation between the amount of enzyme in combination with RNA and the amount present in active form?

It seems possible that the RNA protein particles, which appear so uniform, are in reality very heterogeneous. Possibly specific antisera are the only reagents that will separate them from one another. In any case, this seems worth attempting.

In emphasizing the deletion theory of carcinogenesis I do not wish to imply that no other mechanisms are conceivable. There are many other valid hypotheses, some of which are shown in FIGURE 4. However, I am inclined to attempt to include them all in a common mechanism in which the assortment

Gene mutations: radiation, chemicals

Additions:
Transforming
Principles,
Transduction,
DNA virus



Increase:
 by presence of new
 substrates or inducers.

Deletion:
 by slowing
 reproduction
 relative to
 cell division;
 by competition
 imposed by scarcity.

Addition:
 of virus,
 RNA-type

FIGURE 4. Mechanisms for altering the properties of a cell line (such as carcinogenesis, resistance to drugs, differentiation, and infection) by changing the kind or amount of the cytoplasmic RNA species.

Abbreviations: n—nuclear; c—cytoplasmic; superscript G—assumes DNA prototype; superscript O—assumes no DNA prototype.

The working assumption is that each protein corresponds to a species of cRNA. Acid-soluble nucleotides are not shown.

of enzyme-forming systems in the cytoplasm is altered permanently. I believe that further work on this challenging problem should include the combined techniques of cell fractionation, immunology, and enzyme assay.

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THE GENETICS OF TRANSPLANTATION*

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Grafts within an inbred strain are called isografts; grafts between inbred strains or, more broadly, between any two genetically different individuals of the same species, are called homografts. Whereas isografts of tumor or normal tissue regularly succeed, homografts almost always fail. It is now well established that the failure of homografts is due to genetic differences between donor and host. The genes or genetic loci concerned have been given the name "histocompatibility genes" or "histocompatibility loci."

The analysis of histocompatibility genes requires specialized genetic methods that so far have been applicable only in the mouse.^{8, 12-14, 18, 19} The genetic methods include the use of genes producing visible effects and found to be linked with histocompatibility genes as "markers" or "tags"; the introduction, by appropriate crosses, of histocompatibility genes onto inbred backgrounds to produce "isogenic-resistant" lines; and the use of crosses, especially those in which one parent is from an isogenic-resistant line, to produce hybrid generations. Tumor transplants provide the test for susceptibility or resistance. We shall speak here only of the production and certain limited uses of the isogenic-resistant lines. Suffice it to say concerning the efficacy of the methods taken as a whole, that they permit the identification of individual histocompatibility loci, of the linkage relations of each locus, of the alleles at each locus and, in some cases at least, of the antigenic components or factors characteristic of each allele. However, like other genetic studies with mice, the processes require time; the production and analysis of some of the necessary stocks may require as much as four or five years.

The method of production of isogenic-resistant or IR lines is shown in FIGURE 1, and is described in the legend accompanying this figure. The end result is a pair of strains, A and A.B, essentially identical or isogenic except for a difference at one histocompatibility locus. Because of this one difference each tends to resist grafts from the other. The two lines A and A.B are referred to as a coisogenic pair; or A.B, the synthesized line, may be designated as an isogenic-resistant or IR strain.

Nearly 8 years ago about 125 crosses were set up to produce IR lines. Many of these were lost, and others were discarded because they represented duplications. We now have 23 established IR lines. These are on 11 different inbred backgrounds: A, AKR, BALB/c, C57BL/6, C57BL/10, C57L, C3H, DBA/1, LP, RIII/Wy, and ST.

In nearly every case the IR line is strikingly similar to the inbred line from which it is derived. Coat color and general appearance are the same; idio-

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synocrasies of behavior, such as wildness or tendency to soil the food hopper, are the same; tumor incidence shows no obvious differences (our data on this point are limited). There are, however, three notable exceptions. Strain A.CA carries the gene for fused tail (*Fu*) as well as a histocompatibility gene, both derived from strain CA, both of them foreign to strain A. Strain B10.LP (abbreviation for C57BL 10.LP) carries the gene *A* or agouti, whereas strain C57BL/10 is *a*. Strain C3H.K is *c* or albino, whereas C3H is *C*.

The fact that a gene producing a visible effect was introduced along with a histocompatibility gene at once suggested that the two were linked. This supposition has been confirmed by an analysis of the segregating (even-numbered) generations, and/or by subsequent tests.^{1, 16, 17} In the terminology of the geneticist, the genes *c*, *Fu*, and *A* have functioned as marker genes, tagging the particular piece of chromosome on which the histocompatibility gene is borne. Since we know that the gene *c* is in linkage group 1, *Fu* in linkage group 9, and *A* in linkage group 5, we can infer at once that we are dealing with three separate histocompatibility loci. These have been designated *H-1*, *H-2*, and *H-3*, respectively.

Eichwald and Silmsen⁶ have reported results with skin grafts that have been interpreted as indicative of a histocompatibility locus on the Y chromosome of the mouse.¹⁵

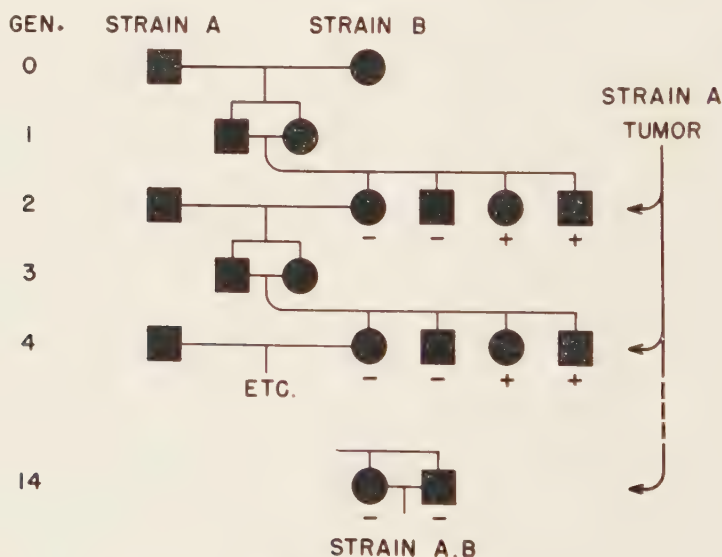


FIGURE 1. Method for the production of isogenic-resistant (IR) lines of mice. In this pedigree chart, A stands for any inbred strain of mice, and B stands for some other strain of mice which may or may not be inbred. Strains A and B are crossed, and an F_2 generation is raised. This and subsequent even numbered generations are inoculated with a Strain A tumor. The survivors (indicated by minus signs) are mated back to Strain A. In generation 12 or 14, two survivors (indicated by minus signs) are mated together to produce IR Strain A.B. This strain carries some single histocompatibility gene derived from Strain B which is different from the corresponding or allelic gene in Strain A. Except for this, Strain A.B is essentially identical or isogenic with Strain A. Grafts made in either direction between Strains A and A.B carry a foreign isoantigen and tend to be destroyed because of this difference at a single histocompatibility locus.

TABLE 1

THE HISTOCOMPATIBILITY AND "MARKER" GENE DIFFERENCES BETWEEN SOME ISOGENIC RESISTANT STRAINS OF MICE AND THE INBRED STRAINS FROM WHICH THEY WERE DERIVED

Inbred strains		Isogenic resistant strains		Differential loci
Designation	Genotype	Designation	Genotype	
A	<i>H-2^afu</i>	A.BY	<i>H-2^bfu</i>	<i>H-2</i>
		A.CA	<i>H-2^fFu</i>	<i>H-2 Fu</i>
		A.SW	<i>H-2^gfu</i>	<i>H-2</i>
C57BL/10	<i>H-2^bH-3^aa</i>	B10 D2*	<i>H-2^dH-3^aa</i>	<i>H-2</i>
		B10.LP*	<i>H-2^bH-3^bA</i>	<i>H-3 A</i>
C3H	<i>H-1^aCH-2^k</i>	C3H.SW	<i>H-1^aCH-2^b</i>	<i>H-2</i>
		C3H.K	<i>H-1^bcH-2^k</i>	<i>H-1 c</i>

* In these compound symbols, the designation of strain C57BL/10 has been abbreviated to B10, and of DBA/2 to D2.

We cannot go here into the methods other than linkage by which the histocompatibility genotype of the IR lines has been determined. Suffice it to say that all except a few of them have now been typed. In the great majority of instances the difference between the inbred strain and its coisogenic mate is at the *H-2* locus.

TABLE 1 lists seven of the isogenic-resistant strains on three different inbred backgrounds, and shows the histocompatibility genotype of each as far as determined, as well as any visible gene differences. The complete histocompatibility genotype of each strain, when ultimately determined, will probably include ten or more histocompatibility loci, just as complete blood typing for any human would involve some ten or a dozen blood-group loci. Of course, the important thing in TABLE 1 is the histocompatibility locus at which the inbred and isogenic-resistant strains differ. This is listed in the last column of the table.

The *H-2* locus has been the subject of a recent review,¹⁰ and we shall not discuss it in detail here. Twelve alleles have been identified by the methods mentioned above, and more will certainly be found (unpublished data¹¹). As originally shown by Gorer,⁷ *H-2* determines not only susceptibility and resistance to transplants, but also the agglutinability or nonagglutinability of erythrocytes by antisera from mice that have rejected transplants. It is both a histocompatibility gene and a blood-group gene. This has made it possible to study the antigens which it determines by classic serologic methods, with the result that a complex system of antigenic components has been demonstrated.^{3, 10, 11}

The *H-1* and *H-3* loci are less fully analyzed. In the case of *H-3*, however, we have evidence for the existence of at least four alleles, and of at least two antigenic factors or components.¹² The allele *H-3^b*, present in strain C57BL 10, one member of the coisogenic strain pair in terms of which this locus is defined, is also present in the related strains C57BR/cd and C57L. Allele *H-1^a*, which is characteristic of strain C3H, is also shared by strains DBA/1 and DBA/2.^{16, 17, 19}

While much of the genetic analysis of these two loci must await the development of additional coisogenic stocks, the availability in quantity of the stocks already established has made possible a number of significant studies. One fact that has emerged is that *H-1* and *H-3* are "weaker" loci than *H-2* in the sense that transplants more easily transgress *H-1* and *H-3* differences between donor and host than they do *H-2* differences. In the case of skin grafts, this was manifest in the form of prolonged survival.⁵ Grafts of normal skin made between strains C57BL 10 and B10.LP, or between C3H and C3H.K, with differences at *H-3* and *H-1*, respectively, survived on the average 24 days or longer. In the same experiment, survival where donor and host differed at *H-2* was about 8 days.

Further evidence of the relative lack of "strength" of *H-3* and *H-1* comes from studies with tumor grafts. Some pertinent data are given in TABLE 2. Case 2 in this table shows the growth of C57BL 10 tumor C1498 in hosts of strain B10.LP. Donor and host in this instance differ at the *H-3* locus. It will be seen that all mice are killed by a dose of 125,000 cells per mouse, and 9 of 11 by a dose of 1000 cells per mouse. This result is indistinguishable from that obtained when the tumor is inoculated in the native strain (Case 1). In fact, if we were to rely only on information obtained from C1498 tested in unimmunized mice, we should be quite unaware of any histocompatibility difference between this particular pair of strains.

When, however, B10.LP mice are preimmunized with macerated embryo from Strain C57BL 10, growth of C1498 is almost completely inhibited (Case 3). In the particular experiment summarized in the table, only 3 of the 43 mice succumbed to the tumor. A potential resistance to the tumor is thus

TABLE 2
THE EFFECT OF IMMUNIZATION WITH DONOR TISSUE ON THE MANIFESTATION OF RESISTANCE TO CERTAIN VIRULENT TUMORS WHERE DONOR AND HOST ARE ISOGENIC EXCEPT FOR DIFFERENCES AT THE HISTOCOMPATIBILITY-1 (*H-1*) AND HISTOCOMPATIBILITY-3 (*H-3*) LOCI

Case number	Donor	Tumor	Host	Tissue used for immunization	Donor and host differ at	Fraction mice dying at different tumor doses			
						Tumor dose 1000×			
						5-1	5 ¹	5 ³	5 ⁶ cells
1	C57BL/10	C1498	C57BL/10	None	No difference	6/10	9/9		
2	C57BL/10	C1498	B10.LP	None	<i>H-3</i>	9/11		11/11	
3	C57BL/10	C1498	B10.LP	C57BL/10 embryo	<i>H-3</i>	1/11	0/10	1/11	1/11
4	C57BL/10	C1498	B10.D2	None	<i>H-2</i>				0/10
5	C3H	E9514	C3H	C3H embryo	No difference			6/6	
6	C3H	E9514	C3H.K	None	<i>H-1</i>			10/10	6/8
7	C3H	E9514	C3H.K	C3H embryo	<i>H-1</i>			0/10	1/10

present in strain B10.LP, but is manifested only where an immunity to the foreign *H-3* antigen of strain C57BL/10 is established in advance.

In contrast to the ease with which C1498 transgresses an *H-3* difference between donor and host, it almost never transgresses an *H-2* difference. For example, Case 4 in TABLE 2 shows a test of C1498 in strain B10.D2 hosts in which there were no deaths among 10 mice implanted with 3,125,000 cells each.

Cases 6 and 7, TABLE 2, reveal a similar behavior of C3H tumor E9514 tested in strain C3H.K. Here the difference is at the *H-1* locus. Again there is progressive growth in unimmunized mice (though here there are some survivors—as many as 50 per cent in some experiments); protection followed immunization with embryonic tissue from the donor strains.

Amos, Gorer, and Mikulska² have similarly shown, by means of implants of a transplantable leukemia into immunized and nonimmunized mice of a segregating generation, that preimmunization will reveal histocompatibility gene differences that would go entirely unsuspected in its absence, at least with a virulent tumor such as they employed.

It seems unlikely that any other histocompatibility loci will be found as "strong" as *H-2*. The fact that *H-2* differences characterize at least 17 of our 23 established isogenic-resistant lines is indicative of the importance of this one locus in determining susceptibility and resistance to transplants. *H-1* and *H-3* may be among the next most important group of loci. In a new series of crosses set up to establish isogenic-resistant lines, with the parent stocks so selected that there is no segregation at *H-2*, we find these loci, as indicated by the associated marker genes, very much in evidence.

The total number of histocompatibility loci in the mouse has been the subject of various estimates based on the ratio of susceptible to resistant animals in a segregating generation. A recent experiment with skin grafts indicated that not fewer than 14 or 15 histocompatibility loci were segregating,⁴ a result not greatly different from earlier studies with transplantable tumors.¹² It should be added that the various sources of error are apt to lead to an underestimate rather than an overestimate.

It is interesting to inquire whether any other histocompatibility loci besides *H-2* are blood-group loci. During a recent visit to the Jackson Laboratory, Bernard Amos of Guy's Hospital Medical School, London, England, kindly performed some red-cell agglutination tests with our coisogenic strain pairs differing at *H-1* and *H-3*, using the methods developed by Gorer and his co-workers⁴ which are the basis of so much of our knowledge concerning *H-2*. The results were negative.¹⁹ This may mean either that these loci do not determine red-cell antigens, or simply that the methods now in use cannot detect them. It will be surprising if a number of genetic loci are not ultimately proved to act in both capacities.

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CYTOGENETIC ASPECTS OF COMPATIBILITY

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The immunogenetic principles of tissue compatibility are now well established. They have been elaborated largely through transplantation experiments with neoplasms of inbred mouse strains,¹⁻³ since tumors are, on the whole, simpler in their isoantigenic growth requirements than are normal homografts. A recent critical summary by Gorer⁴ reviews the subject from the serologic aspect. Orientation regarding linkages of histocompatibility factors in the mouse may be gained from several papers by Snell and his co-workers;⁵⁻⁷ the pertinent facts have also been considered against the general background of tumor immunity⁸ and tissue genetics.⁹⁻¹¹

Genetic Rules and Inconsistencies in Tumor Transplantation

Snell⁶ has formulated four "laws" governing homograft survival as follows: (1) tumor transplants originating in an animal of an inbred strain are compatible with all individuals of the same strain; (2) "tumors transplanted within the species, but outside the strain of origin, fail to grow, or grow temporarily and then regress"; (3) F_1 hybrid animals between strains are susceptible to grafts from either parent stock; and (4) neoplastic tissues arising in an F_1 hybrid can grow in all similar hybrids, but not in either parental genotype.

These generalizations apply also to normal tissues, the essential prerequisite being that the recipient host match the transferred cells with respect to their isoantigens. If, on the other hand, a graft carries one or more antigens, for which the recipient has immunologically different alleles, the implant evokes an antibody response.

In a classic transplantation test, the percentage of successful "takes" in backcross or F_2 hybrids follows Mendelian ratios, and thereby indicates the number of segregating histocompatibility genes. Whether or not statistical agreement with Mendelian expectation can be translated literally into genetic terms depends on a number of variables affecting antigenic efficiency. Does a 25 per cent "take"-percentage in a backcross test really signify a 2-gene difference between graft and host, or is it the composite result of promoting and retarding influences in the same experimental group? Taking into account the role of graft site, host age and sex, and maternal influence in such a test, particular combinations of these factors allow "takes" that range all the way from 5 to 50 per cent in the same genetic system.⁹⁻¹² The ceiling of 50 per cent is clearly determined by a strong isoantigen. The average of 25 per cent indicates the need for at least a second weaker antigen. Some "false negatives" may be ascribed reasonably to nongenetic factors.

Here it should be stressed that even the genetic determinants of graft failure need not all be immunological. Hereditary metabolic or hormonal requirements by which the implant differs from the recipient could mimic antigenic segregation in test hybrids. Thus, all estimates of the total number of histo-

compatibility loci in a species, based merely on transplantation data, should be subjected to eventual serologic proof.

A conservative estimate of the total number of loci that determine tissue compatibility between mouse strains is fourteen.¹³ Three of these histocompatibility factors (H factors) have been localized definitely on autosomal linkage groups I, V, and IX.¹⁴⁻¹⁷ Tumor and skin graft data of several authors lend hypothetical support to the existence of similar factors also on the X and Y chromosomes.^{12, 13, 18-21}

Some of the H factors influence growth more than others, the H-2 region being by far the strongest.²² It is for this reason that H-2 has been most thoroughly explored in serologic terms. Gorer's original saline hemagglutination method²³ has been perfected toward a highly sensitive dextran-enhanced procedure that permits determinations of isoantibody down to dilutions of 1:16,000.^{24, 25} A further immunogenetic refinement makes use of isogenic-resistant hosts⁶ for the production of isoagglutinating test sera. Such hosts differ from the donor tissue at a given H region, but not necessarily in a single antigen.

The known antigenic components of the H-2 system in various mouse strains obtained by Hoecker, Counce, and Smith,²⁶ Hoecker,²⁷ Amos, Gorer, and Mikulska,²⁸ and by Gorer and Mikulska are tabulated in Gorer's recent review.⁴ This system is rapidly approaching the cattle blood groups in its complexity. The A-strain cell, for example, carries at least 12 H-2 antigens on its surface. What had appeared, from transplantation experiments, to be a simple locus with a number of allelic forms is, in reality, a chromosomal region with a large number of closely linked pseudoalleles. True allelism holds for some of the "genes" of the H-2 system, and multiple allelism has been found for the series *D-Db-d* and *E-Ed-e*.

Crossing-over within the H-2 region has been determined with certainty on 3 occasions,²⁸ and with probability in one further experiment.¹⁷ FIGURE 1 depicts the 3 cases detected in Gorer's laboratory: 2 resulted from a cross between A and C57BL mice, and the third from the cross BALB C \times C57BL. *Ed* is shown between *D* and *K*, but its true location, like that of *F*, is not yet established. *C*, on the other hand, must lie between *D* and *K* (P. A. Gorer, personal communication).

D, *E*, and *K* are drawn into the simplified schematic representation, since they served as marker antigens. Additional H-2 antigens that were studied in the crossover mice either segregated with *D* or with *K*, and another appeared to be intermediate, like *C*.

To obtain further data on the intraregional alignment of the H-2 factors, crossing over between *D* and *F* has been sought in individual hemagglutination tests of some 400 backcross hybrids at our laboratory, but to date no such crossover has come to light. Either the linkage is very close or the antisera used are too complex for this type of test. It was originally thought that the sera contained antibodies against only 1 or 2 factors, but, in practice, several factors can be detected simultaneously. This technical difficulty cannot be resolved through the use of isoresistant line pairs; however, it can be minimized by producing the antisera in certain hybrids. For example, the

antisera C3H anti-DBA 2 may contain up to 4 "new" components in addition to anti-*D* and anti-*F*. Absorption with C57 cells removes anti-*F*, together with one of the "new" antibodies, but it leaves behind as many as 3 components besides anti-*D*, necessitating multiple preparatory absorptions. If, on the other hand, the serum is produced in a C3H \times C57 hybrid, the first absorption is eliminated and clearing with tissues from only 2 other strains may suffice. Use of an incompletely cleared antibody favors the chance that crossovers will be missed. Unless this is allowed for, and until the H-2 region is more completely mapped, estimates of crossover frequencies are premature.

In amassing these immunogenetic facts, strain-specific mouse tumors have been the principal experimental tool. The general conclusions are not invalidated by "progressive" phenomena, which render many neoplasms autonomous with regard to Snell's second transplantation "law" (see above).⁶ A recent endeavor²⁰ to relate this unruliness to virulence, growth rate, or growth in ascitic suspension, cannot be considered fertile, hinging, as it does, on the dichotomy of "good" and "bad" tumors. A good tumor is defined as one that behaves in tune with its own and the host's histocompatibility genotype; it is not influenced by "accidental factors in the environment." The latter are

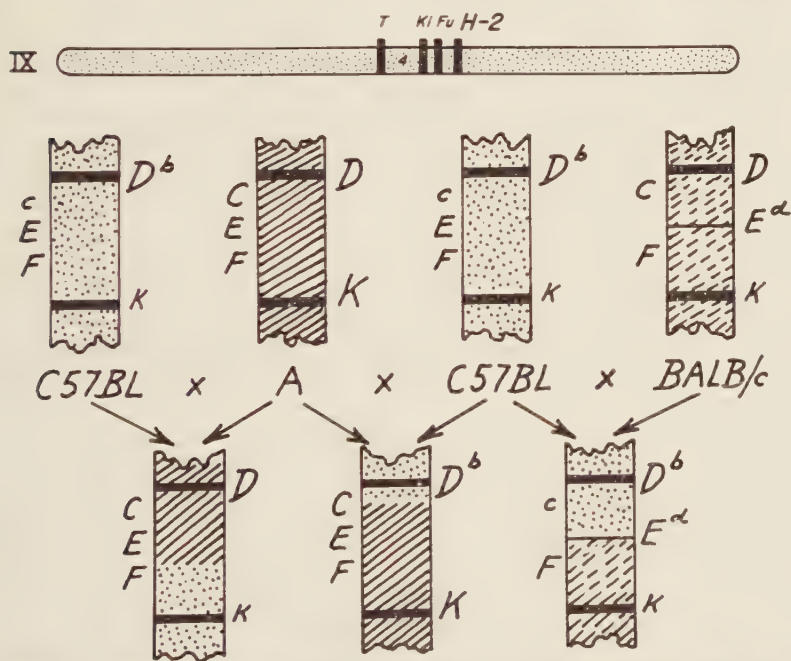


FIGURE 1. The histocompatibility 2 (H-2) region and the crossovers within it. The chromosome map shows the H-2 region on linkage group IX of the mouse in relation to the dominant marker genes T (brachyury), Ki (kinky), and Fu (fused tail). Below this linkage map are diagrammatically shown some H-2 alleles and pseudoalleles of 3 inbred strains and the antigenic rearrangements due to crossovers that have occurred in hybrids between these strains.

thought to play a relatively greater role in the annoying nonconformity of bad tumors. The worst culprits are the so-called universal or nonspecific tumors.

Among the most "virulent" rapid growths may be found strain-specific (for example, L # 2, 6C3HED original line, TA3 1) as well as "universal" types (for example, Krebs 2, Ehrlich, S180) covering a broad histological range. The same incongruity applies to ascites tumors as a group, the ease

TABLE 1
ISOANTIGENICITY OF NEAR-DIPLOID MOUSE TUMORS

Tumor	Geno- type of origin	Modal chromosome number	Graft speci- ficity	H-2 antigens								
				A	C	D	E	F	H	K	D ^b	Q
TA3 (1950)	H-2 ^a	40	S			++				++		
TA3 (1956)	H-2 ^a	43	L	++	++	++	++	++		++		
6C3HED (until 1956)	H-2 ^b	40	S	+-		0	+	0	++	+	0	0
6C3HED (1957)*		41-43	L									
A # 2	H-2 ^a	44	S	++		++	++	++	++	++	0	0
DBA/2	H-2 ^d	42	S	0		++	E ^d	++	++	0	0	
DL1	H-2 ^d	~40	S			++	0	++	++	0	0	
QL1	H-2 ^a	~40	S				++	++			0	++
EL24	H-2 ^b	~40 and 80	S	0		0	++	++		0	++	0
4 Spont. mammary Sarcoma I	H-2 ^k	~40	S			0	++	++	++	++	0	
	H-2 ^a	42-44	L			++	++		+	++		

S = strictly specific

L = limited nonspecificity

~ = approximate

* Antigens variable, not consistently weak

++ = strong antigenicity

+ = antigen present

0 = negative test, as expected on genetic grounds

TABLE 2
ISOANTIGENICITY OF HETEROPLOID MOUSE TUMORS

Tumor	Genotype of origin	Modal chromosome number	Graft specificity	H-2 antigens								
				A	C	D	E	F	H	K	D ^b	Q
Ehrlich (Lettre)	unknown	45-47	N			-		-		-		
Ehrlich (Klein)	unknown	78	N		-	-		-		-		-
Krebs 2	unknown	75	N			-	±	-		±	-	
TA3/2	H-2 ^a	74	N		-	-		-		-		
A # 1	H-2 ^a	~80	L			+	±	+		±	0	
6C3HED/2	H-2 ^k	~78	L			±	±			±		
MC1M	H-2 ^k	67	L			0	±	0	++	±	0	
S37 (solid in DBA/1)	unknown	40 and 80	L	++	-	-			++	+	-	
P288	H-2 ^d	~80	L			++	E ^d	++	++	0	0	
15091a (solid, A)	H-2 ^a	~80	L			++			++	++		
S180 (solid, A)	unknown	~80	L or N		+	+	-		+	-		

N = widely nonspecific

L = limited nonspecificity

~ = approximate

++ = strong antigenicity

+ = antigen present

± = antigen very weak or absent

0 = negative test as expected on genetic grounds

- = negative test

of conversion from the solid to the ascitic form being quite unrelated to histocompatibility in our experience with a broad spectrum (TABLES 1 and 2).

The "accidental factors" responsible for genetic inconsistencies are, however, amenable to experiment, if one considers neoplastic cell populations as fluctuating cytogenetic mosaics³⁰ and integrates transplantation tests with cytologic³¹⁻³³ and serologic analysis.³¹⁻³⁶ This approach to altered graft behavior holds out some promise for the eventual understanding of immunologic deviationism among tumors.

Karyotypic Drift in Neoplasms

The apparently wide distribution of antigenic "loci" throughout the mouse idiogram, as shown by transplantation studies, pseudoallelism, and crossing-over within the H-2 complex, emphasizes the potential immunogenetic consequences of gross chromosome changes within a graft. Since chromosome aberrations are frequent in cancer cells,³⁷ the influence of nuclear anomalies on the differentiation of normal isoantigens deserves critical attention. Antigenic stability in cells with severely unbalanced chromosome sets would, indeed, be a genetic paradox. As one might predict, karyotypic changes in malignant tissues are often accompanied by quantitative alterations in antigenic content. Certain normal isoantigens may disappear altogether.³²⁻³⁶ This finding is in harmony both with the concept of antigenic "simplification"³⁸ and with the occasional appearance of serologically "new" properties in neoplasia.^{39, 40}

Improvements in cytological techniques, such as the aceto-orcein squash method, have made mammalian chromosomes in general, and tumor karyotypes in particular, more accessible to precise study. A vast literature has accumulated on numerical and structural chromosome anomalies, especially in ascites tumors.^{9-11, 37} These free suspensions of cancer cells in peritoneal exudate⁴¹ facilitate immunogenetic analysis. Their advantages include: precise cell dosages for inoculation and absorption,²⁵ possibility of quantitative growth measurements in the presence or absence of immune reactions,⁴² ease of cytological squash preparations, and suitability for critical isolation and comparison of single cell clones.^{30, 43} Furthermore, ascites cells expose their naked surfaces to circulating isoantibody and to attack by the host's defensive cells. Dependence on vascular supply for survival is still another variable eliminated from experiments with ascites tumors. Hence, their immune responses are less complicated by host-environmental influences (accidental factors²⁹) than is the reactivity of solid neoplasms.

The availability of a broad ascites tumor spectrum has promoted cytogenetic population analysis of the nature, range, frequency, and stability of nuclear changes. How permanent are the stem lines? How viable are the products of their mitotic errors? Can general karyotypic features (aneuploidy, for example) be correlated with such clear-cut cell functions as antigenicity?

The mitotic anomalies that tend to create genic imbalance in all cancer tissue have been thoroughly investigated in the mouse,⁴⁴⁻⁴⁵ the rat,^{46, 47} in man,⁴⁸⁻⁵⁰ and in the Chinese hamster.^{51, 52} The most frequent chromosome numbers in all the mammalian neoplasms examined are somewhat hyper-

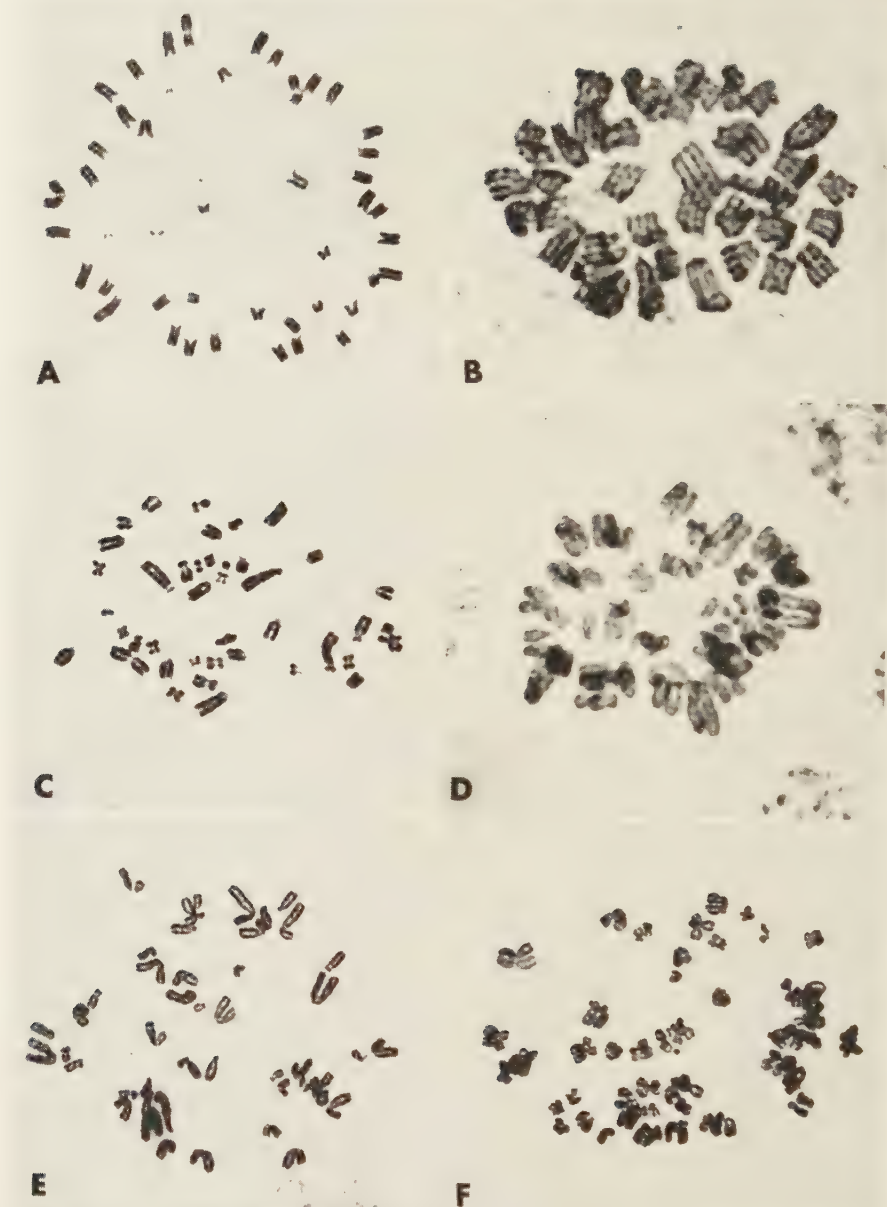


FIGURE 2. Metaphases seen in aceto-orcein squash preparations of several ascites tumors. (A) Hyperdiploid cell of the Ehrlich mouse carcinoma with 46 chromosomes, including 3 "minutes"; at 12 o'clock a unit with secondary constriction, and at 9 o'clock a metacentric chromosome ($\times 1200$). (B) Four stranded chromosomes produced by endoreduplication in a malignant mouse lymphocyte ($\times 2500$). (C) Typical cell of the Yoshida rat sarcoma; 42 chromosomes ($\times 1400$). (D) Endoreduplication metaphase in the Yoshida rat sarcoma ($\times 1400$). (E) Diploid metaphase from a human ovarian carcinoma ascites ($\times 1000$). (F) Four stranded diplochromosomes in a cell from the same human ovarian neoplasm shown in E ($\times 1000$). (Minutes are very small new chromosomes significantly shorter than the shortest normal chromosome in the characteristic idiogram of the species. Some minutes apparently contain essential genic material, since they persist in certain tumor stem cell lines during several years of serial transplantation.)

diploid or hypotetraploid. However, many tumors, especially the lymphomas and spontaneous mammary tumors, have, at least superficially, diploid modes.^{30, 32, 37, 44, 50, 52-54}

Mechanisms for polyploidization have been described in great detail.^{44, 49} They include endomitosis proper, endoreduplication characterized by four-stranded chromosomes, division failure due to unipolar spindles, and possible fusion in binucleate cells. The universality of such occurrences in different species is evident from FIGURE 2A-F, which shows normal mitosis alongside endoreduplication in tumor cells of the mouse, the rat, and in man. A characteristic endoprophase can be seen in FIGURE 3C, and three endometaphases in FIGURE 4B. Extreme cases of polyploidy (FIGURE 3A) are unimportant population components. On the other hand, new stem-cell types with unbalanced hypotetraploid numbers often arise in the wake of polyploidization (FIGURE 3B).

Aneuploidy (FIGURE 3E) of a numerically less pronounced type may arise from multipolarity (FIGURE 3D), but more often from nondisjunction, or lagging (FIGURE 3F), to mention but a few of the common spindle errors.

Far more important from a genetic viewpoint than mere chromosome doubling are the gross structural differences between the normal diploid idiogram of a host species and the stem lines of corresponding tumors. These abnormalities include "new" metacentrics (FIGURE 2A), minutes (FIGURES 2A, 3E, and 4A), dicentrics, and rings.³⁷

Levan's painstaking chromosome length measurements^{37, 55} have uncovered "cryptostructural" karyotypic remodeling of fundamental significance. While the diploid mouse tumors examined by this investigator had idiograms morphologically almost indistinguishable from the normal mouse karyotype, all the near-tetraploid neoplasms exhibited greater extremes in chromosome length. The diploids apparently had not undergone translocations of the more asymmetric variety. In the polyploids, on the other hand, intrachromosomal rearrangements were extensive. This enhanced cytogenetic flexibility of near-tetraploid tumors furnishes the "mutant" resources and "escape gaps" for survival under adverse selective pressures, as it does in polyploid plants.⁵⁶

The magnitude of numerical departure from the diploid norm is less important in the present context than genic imbalance resulting from aneuploidy and cryptostructural position effects. Reasonable constancy of a balanced genome would foster the integrity of normal isoantigens. Even a minor nuclear aberration can undo this essential balance, depending on the genes involved.

Embryological observations of Fankhauser and Humphrey⁵⁷ are pertinent here. In amphibian development, addition of a single chromosome to the diploid idiogram creates disturbances as far-reaching as near-triploid imbalance.

Chromosome Balance and Antigenic Differentiation

A functional relationship between the modal chromosome constitution of neoplasms, their transplantation range, and their isoantigenic content has come to light from our extended survey of mouse tumors.³⁰⁻³⁶

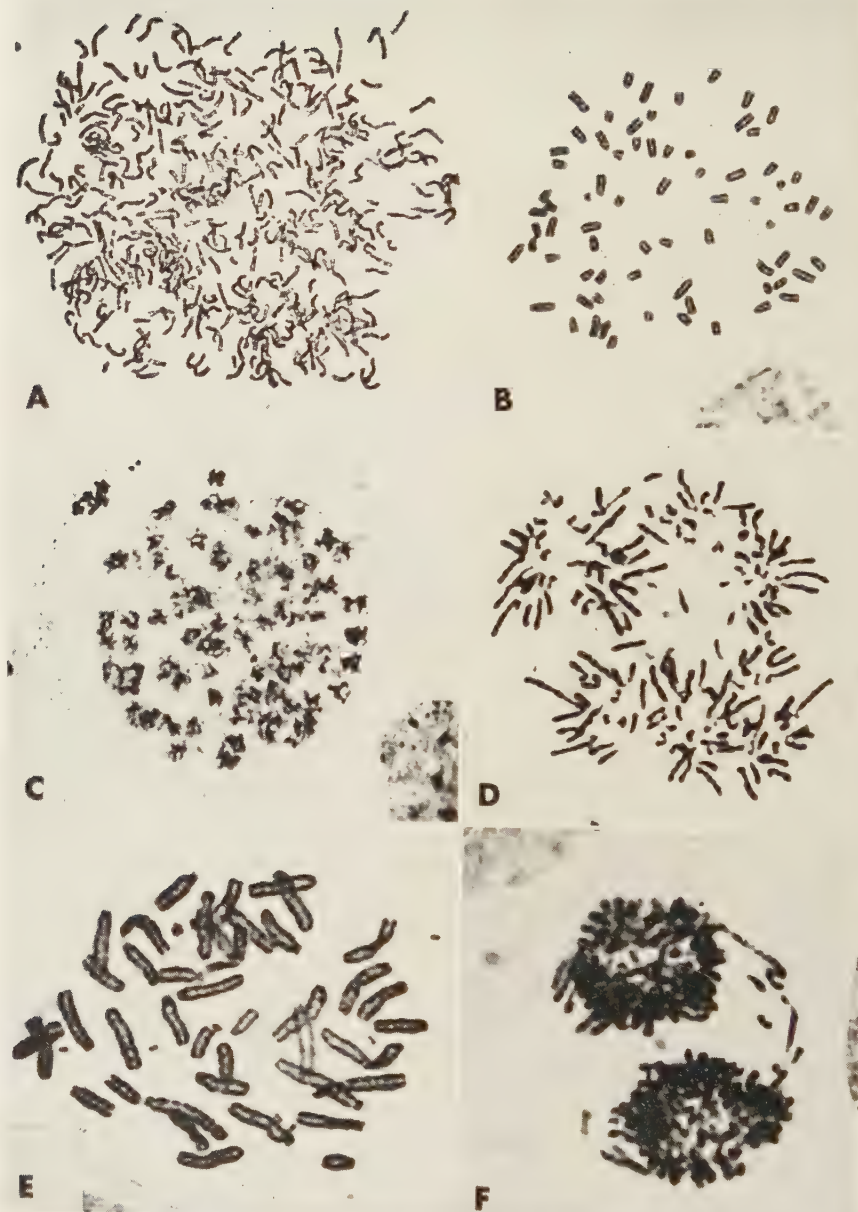


FIGURE 3. (A) Prophase in a high polyploid of the Krebs 2 mouse ascites carcinoma ($\times 1000$). (B) Metaphase of a stem cell from a clone of the hypotetraploid Ehrlich mouse sarcoma, with 72 chromosomes ($\times 1000$). (C) Characteristic endoprophase, showing the diploidy of condensing chromosomes and intactness of the nuclear membrane, in a cell of the MC1M mouse fibrosarcoma ($\times 1530$). (D) Tripolar anaphase in the Yoshida rat sarcoma ($\times 1530$). (E) Hyperdiploid metaphase in the mouse Sarcoma I (A strain), showing 6 "minutes," some of them clearly double ($\times 1530$). (F) Abnormal anaphase from a human ovarian ascites carcinoma, showing a bridge and lagging ($\times 1530$).

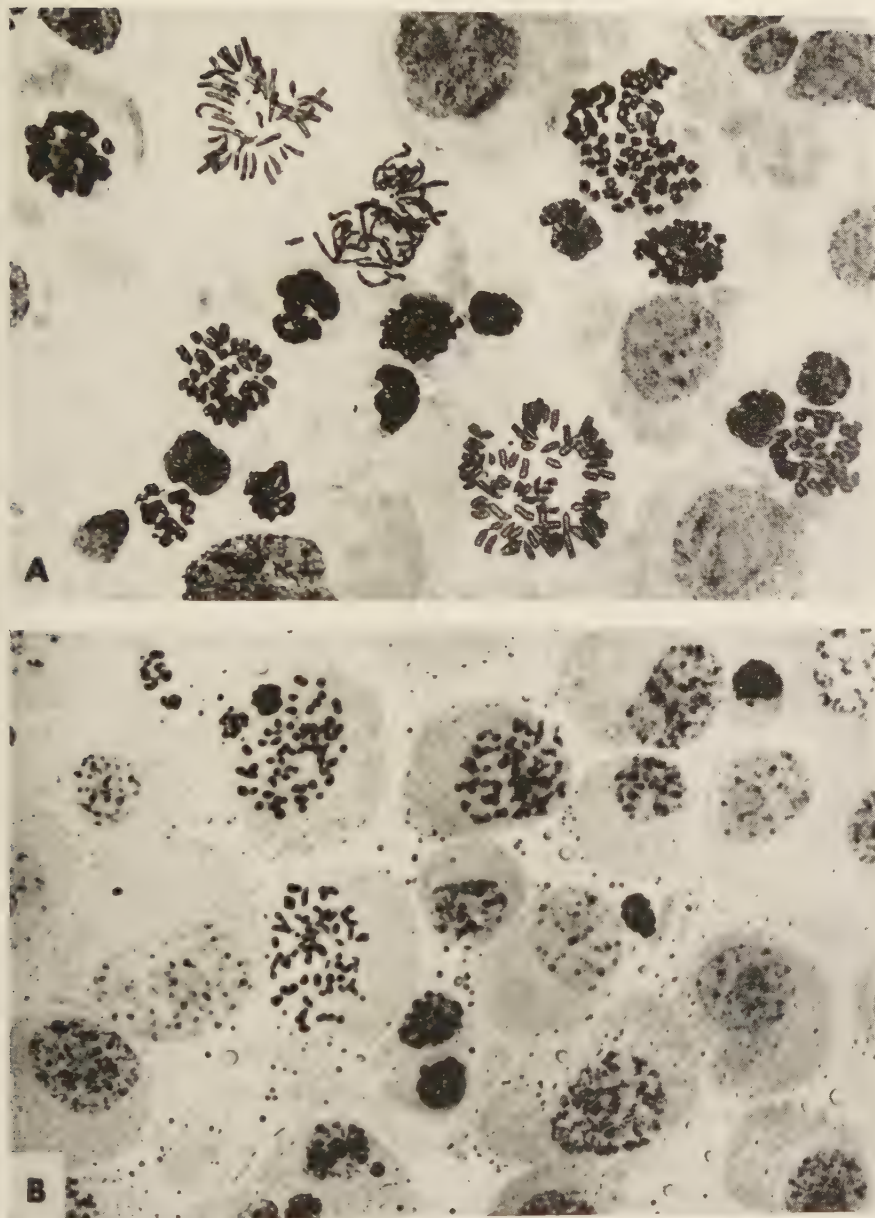


FIGURE 4. (A) Typical field from an aceto-orcein squash of the mouse Sarcoma I ascites. Sample taken 17 hours after the intraperitoneal injection of 5γ of colchicine; that is, prior to the possible production of heteroploids by colchicine treatment. The majority of metaphases is near diploid, but the plate near the lower center is approximately tetraploid. A "minute" may be seen in the latter location, and another in the metaphase at the upper left. Several plates show pronounced mitotic effect. The tumor cell population contains an appreciable number of host leukocytes ($\times 600$). (B) Typical endometaphases in the MC1M mouse fibrosarcoma ascites ($\times 600$).

TABLES 1 and 2 bring up to date the salient observations for 14 near-diploid and 11 heteroploid mouse neoplasms. Historical and histological data for most of these tumors are listed by Dunham and Stewart.⁵⁸ The mouse strains of origin include A He, A St, C3H St, C3H_f He, DBA 1, DBA 2, C57BL, and unknown heterogeneous stocks. Graft specificity was tested in the above inbred strains and in Swiss mice. Most of the neoplasms were studied in the ascites form, which facilitated chromosome counting and serologic absorption. In the determination of antigens, the dextran procedure of Gorer and Mikulska was followed.²⁴

It should be stressed that most of the near-diploid tumors in TABLE 1 (except the 1956 samples of TA3, and Sarcoma I) lacked obvious chromosomal innovations. Karyotypic changes were, however, apparent in practically all the more heteroploid material of TABLE 2.

The over-all consistency of differences between the two broad categories of tumors is clear at a glance. Most of the diploids ($2n = 40$ in the mouse) and near-diploids cannot grow progressively outside the rigid compatibility limits determined by the mouse genotype in which each originated. Aberrations of the modal chromosome number, ranging from slight hyperdiploidy to erratic polyploidy, favor immunological indifference, especially if they involve "new" chromosomes. This is true for Sarcoma I (FIGURES 3E and 4A), which furthermore contains about 9 per cent of hypertetraploid cells, and for the Lettre Ehrlich ascites (FIGURE 2A). All degrees of nonspecificity occur. Heteroploid tumors, some of which are sublimes of originally strain-specific diploid tumors, may give lethal takes in only one or two foreign strains, or they may be completely indiscriminate in their host requirements. Virulence or length of transplantation history have no important bearing on host specificity.

These cytological and transplantation results are in fair agreement with tests for the various H-2 antigens; that is, significant weakening of some but not other isoagglutinins often accompanies heteroploidy. Three of the so-called universal tumors lack detectable H-2 antigens completely. Although it is tempting to speak of "antigenic simplification" in such malignant cells, this phrase has a precise meaning only as it refers to a definite test standard, in this case, the red cell surface. Compared to normal red cells, many neoplasms lack certain agglutinogens. On the other hand, new antigens called "X," demonstrable by protective antibodies, have been discovered in several lymphomas.^{39, 40} Some mouse strains are refractory even to one or another universal tumor; for example, Sarcoma 37, which lacks all the common H-2 antigens.

Among the cytogenetic mechanisms responsible for the altered histocompatibility of heteroploid tumor cells are the following possibilities. A whole chromosome or fragment carrying an H factor may be lost. Translocations and consequent position effects on the antigens are conceivable. However, in the crossovers shown in FIGURE 1 the dislocated antigens did retain their normal function. Known cases of genic interaction in blood-group synthesis^{59, 60} imply that the loss or multiplication of any synergistic loci could alter the antigenic end products or the immunologically effective organization

of the cell membrane. The enlargement of cell volume by polyploidy and, perhaps, by certain individual chromosomes⁶¹ reduces the surface volume ratio, at the same time increasing diffusion distances. Consequently, the antigenic sites on the cell membrane are fewer per unit mass, and the antigen-to-antibody proportions necessary for growth inhibition are upset. In the absence of cytological markers, particularly for the important IX chromosome, these possibilities must remain hypothetical.

The relationship between chromosome balance and histocompatibility has been substantiated by the mouse-tumor experiments of Sachs and Gallily,⁶²⁻⁶⁴ Mitchison,⁶⁵ and Gorer.⁴ Palmer⁶⁶ did not record a single diploid cell in over 700 metaphase counts of the subtriploid V2 rabbit carcinoma (in the rabbit, $2n = 44$) and ascribes the tumor's indiscriminate transplantability to the observed aneuploidy. Progressively growing homografts were obtained at the Sloan-Kettering Institute, New York, N. Y., with the human HeLa cervical carcinoma, which is hypotetraploid.⁶⁷ Moore, Southam, and Sternberg⁶⁸ also have reported on the neoplastic nature of homografts of "normal" human epithelial cultures which, according to Levan,⁴⁸ contain many nuclear anomalies.

Even heterografts of aneuploid tumors may be partially compatible. This is borne out by the lethality of certain unbalanced human tumors for mildly cortisonized rats¹⁵ and by the long-term survival of the Ehrlich mouse ascites tumor in serial rat or hamster passages. Heterologously propagated tumors may respond to their foreign hosts by alterations in their stem-cell types. Ising,⁶⁹ for example, encountered a stable drop from 80 to 75 chromosomes for the Ehrlich mouse carcinoma when transplanted in hamsters.

Koprowski *et al.*⁷⁰⁻⁷¹ used repeated fetal mouse-passage followed by intracerebral infant passage to induce tolerance⁷² of rat hepatoma AH130. Later they established this tumor as an ascites in untreated adult mice. Here, the neoplastic cell population became exceedingly heterogeneous, with a hypotetraploid chromosome mode around 70 ($2n = 42$ in the rat).

Regarding the species identity of such "adapted" tumors, caution is indicated. Since rat and mouse chromosomes are morphologically very distinct (FIGURES 2A,C) cytology can perform a decisive service, as it did in the marrow chimeras studied by Ford *et al.*⁷³

We participated in a current attempt of Koprowski and Thies to adapt the near-diploid Yoshida rat sarcoma⁷⁴ toward progressive growth in Swiss mice. So far, this has been impossible to achieve, whereas the more heteroploid AH130 did respond to "adaptation." Some of our observations are presented in TABLE 3. The first attempt (line 2) appeared to be superficially successful, but on closer scrutiny we realized that the mouse deaths were due to an unidentifiable anaplastic mouse tumor, in which no rat chromosomes or antigens were detected. When this tumor was returned to rats, a very small residue of surviving rat cells reappeared, and eventually caused the death of these animals (line 3). In a second attempt, now carried through more than 25 mouse passages (line 4), the integrity of the rat idiogram remains unchanged; however, the rat tumor invariably regresses in all the mice, and must be transplanted every 5 days in order to be maintained.

It is of special interest to note that the Krebs 2 and other heteroploid mouse

TABLE 3
EVIDENCE AGAINST "MOUSE ADAPTATION" OF THE YOSHIDA RAT SARCOMA

Tumor line	Lethal for mice	Total meta- phases analyzed	Chromosome type		Per cent ploidy distribution			Rat* antigens	Mouse† antigens
			Rat %	Mouse %	s	2s	4s		
Yoshida tumor in rats	—	271	100	0	96	3	1	+++	—
Y "mouse adapted" (first attempt)	+	834	0	100	0	88	12	—	+
Y "mouse adapted, back to rat"	+	232	1	99	1	75	24	n.t.	n.t.
Y "mouse adapted" (second attempt)	—	2765	100	0	97	2	1	n.t.	n.t.
Krebs 2 in mice	+	161	0	100	2	79	19	—	+

* Mouse (C57 or A) prepared with 5 intraperitoneal injections of 1 cc. citrated whole rat blood; the first 3 injections were given at 3- to 4-day intervals, followed by a rest of 10 days; then a second course of 2 injections 3 to 4 days apart. The tests were hemagglutination tests after absorption of the antisera with tumor cells.

† Sprague-Dawley rat immunized against mouse blood by procedure like that above.

tumors tested (MC1M, Ehrlich, TA3 2) reacted very feebly with a rat anti-mouse serum (line 5). By comparison, 2 near-diploid strain-specific neoplasms were strong absorbers of heterologous hemagglutinins. Heteroploidy, therefore, tends to despecify mammalian cells, not only with regard to their iso-antigens, but also in some species properties.

We should not, however, overstress the agreement of this finding with the relationship between ploidy and histocompatibility. It may be well to see what can be learned from certain exceptions.

The tumors of TABLES 1 and 2 are, at least superficially, more consistent in their transplantation behavior than in their antigenic titers. No mouse tumor with an exactly diploid mode can grow progressively (that is, it cannot kill the host) across an H-2 barrier. All of the diploids are H-2 specific, serologically and in their host restrictions; they are incompatible even across the weak H-2_q barrier in DBA 1 mice. On the other hand, no grossly hyperdiploid or near-tetraploid tumor has respected the H-2 barrier. The exceptional polyploid, lymphatic leukemia P288, cannot grow in any foreign strain other than DBA 1. Compatibility with DBA 1 appears to be a sensitive first sign of antigenic degradation. This confirms the transplantation data of Snell *et al.*⁷⁶ in H-2_q mice.

Other exceptional tumors are TA3 (1956), Sarcoma I, and MC1M. All three have a fairly wide transplantation range despite their strong H-2 agglutinins. This raises the question whether serologic typing represents the cell population as a whole, and whether small residues of antigenic "mutants" are responsible for the conflicting transplantation data. The cytological prerequisites for such mutants are suggested by new chromosomes, cryptosstructural rearrangements, and aneuploidy in all three of these exceptional tumors. Sarcoma I, for example, studied by Ford⁷⁶ and in our laboratory, regularly shows one or more "minutes" (FIGURES 3E and 4A), an occa-

sional metacentric, and a tendency toward a secondary polyploid mode. As has been shown by experiment,³⁵⁻⁷⁷ such innovations may yield adaptable stem lines for immunoselection.

When a tumor is propagated within the inbred strain in which it originated (that is, in the absence of incompatibilities), selection is likely to favor the balanced diploid. In the presence of antigenic antagonisms, selective pressures would change, favoring the more flexible heteroploids. Klein, Klein, and Révész⁷⁸ have shown that an exceedingly small compatible fraction of the inoculated cell population, amounting to only 20 cells among 50 million incompatible cells, can be selected out of the whole. This happens despite simultaneous destruction of the overwhelming majority. Thus, a strong hemagglutinin titer tells us nothing about an elusive nonspecific cell minority in the neoplastic population. Other population elements that might confuse the meaningfulness of antigenic titers are the host leukocytes and histiocytes, which are often numerous in tumors such as MC1M (FIGURE 5A).

The foregoing arguments and the serologic data of TABLE 2 minimize recent conclusions reached by Feldman and Sachs⁷⁹ in their serologic comparison of 3 mouse tumors, 2 of which we have found rather exceptional in our series of 25. The 6C3HED lymphoma is one of our weaker reactors in the hyperdiploid category, and has lately shown signs of extended homotransplantability. The MC1M gives unexpectedly strong reactions and secretes a viscous mucopolysaccharide that may protect the cell surface against isoantibody. We agree with these authors that, in this instance, the loss of strain specificity could be "due to an increase in the capacity to resist iso-immune response." Clones derived from cells surviving in immunized mice would help settle this question. FIGURE 5A shows healthy MC1M sarcoma cells unaffected by the proximity of numerous host elements. However, totally nonspecific tumors may turn the tables on the host's cellular defenses and cannibalize leukocytes, as does the engorged Ehrlich cell in FIGURE 5B.

Cellular Sites of Isoantigens

The knowledge that histocompatibility antigens are controlled by definite loci on certain linkage groups and are often disrupted by chromosomal imbalance reveals nothing about the morphologic framework of tissue immunity. Where in or on the cell are the specific molecules responsible for the homograft reaction, and how do they couple with antibody (be it circulating or delivered by immune lymphocytes)?

Obviously, polyploidy increases cell volume and diffusion distances. Individual excess chromosomes may accomplish similar enlargement.⁶¹ Tetraploid lymphoma cells that we have measured *in vivo*³⁵ had almost twice the volume of corresponding diploids, but their surfaces were only 1.6 times larger. If there is a proportional reduction or excessive crowding of antigenic sites on the plasma membranes of heteroploid cells, antibody binding might be altered quantitatively, and some antigens could be displaced altogether.

In a recent publication⁸⁰ Billingham, Brent, and Medawar conclude that the antigens responsible for skin transplantation immunity "reside wholly in the nuclei of cells" and are deoxyribonucleoproteins. Cytoplasmic prepara-

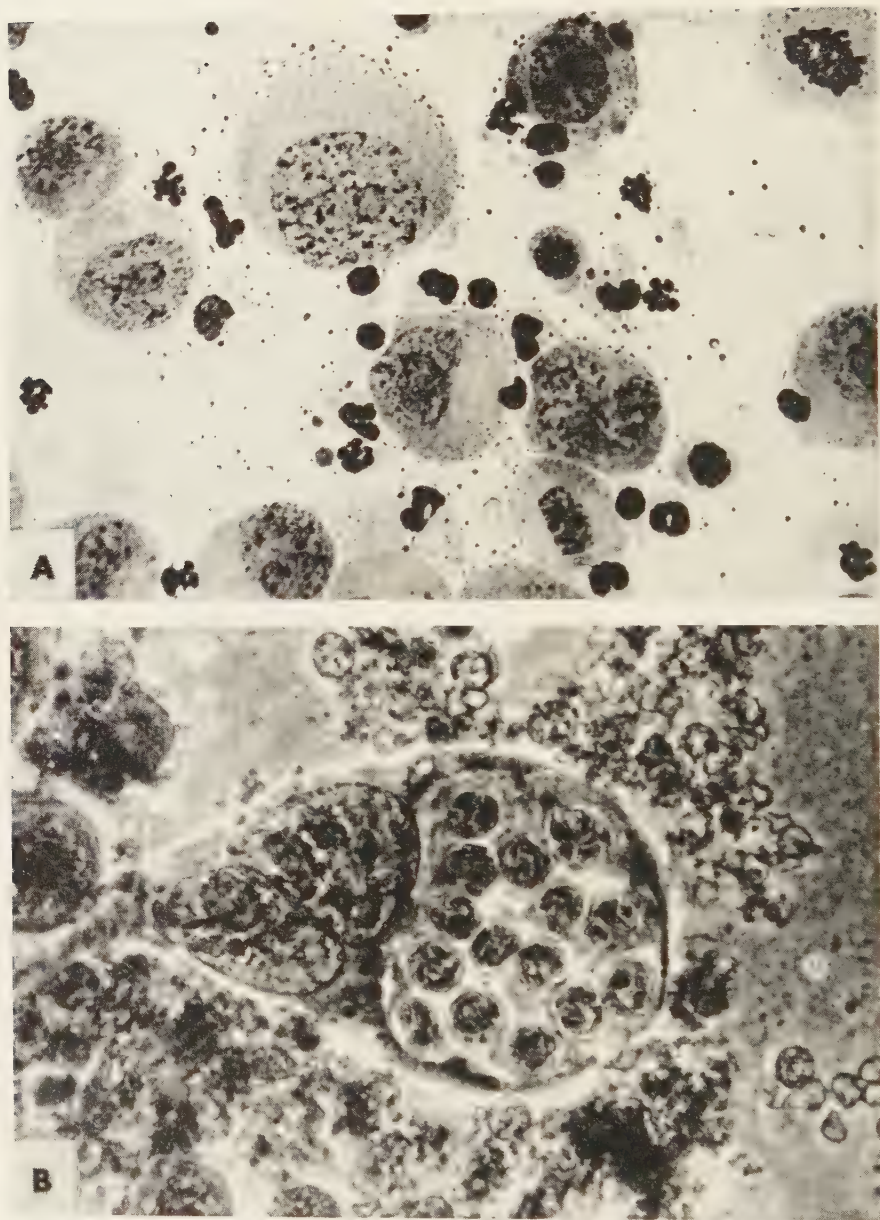


FIGURE 5. (A) Aceto-orcein squash of the MC1M mouse fibrosarcoma ascites, revealing large numbers of the host leukocytes that may be a confusing source of isoantigens in absorption tests. Single passage in C57BL mouse ($\times 600$). (B) Phase-contrast photomicrograph of a living cell from the Ehrlich mouse carcinoma engorged with polymorphonuclear leukocytes. The two large nuclei and prominent nucleoli in this cell suggest that it is a neoplasm rather than a host macrophage ($\times 1200$).

tions and ribonucleic acid (RNA) were ineffectual in the experimental production of accelerated skin sloughing. However, the integrity of the plasma membrane, which may be all-important for retention of specificity, would have been disrupted in these antigenic preparations more severely than the other fractions. While the proposed antigenic role of deoxyribonucleic acid (DNA) reinforces the functional connection between chromosomes and histocompatibility, it should be remembered that the immunologically indifferent polyploids have more DNA and a greater variety of DNA than do the diploids. From experiments with the simple cytotoxicity test of Gorer and O'Gorman,⁸⁴ an indication that the permeability of the surface is directly affected by antibody in the presence of complement is obtained. Fluid rapidly enters many cells that swell and lyse.

We need not, however, insist that isoantibody interferes with a homograft mainly on the cell surface.²⁹⁻³¹ Globulin uptake and transfer to cytoplasmic or intranuclear sites of binding may be accomplished by mechanisms differing in various tissues, including phagocytosis (FIGURE 5B), pinocytosis, continuity of the endoplasmic reticulum with pores in the plasma membrane, and porosity and lobation of the karyotheca. The layers, perforations, and thickness of plasma and nuclear membranes (120 and 230 Å., respectively) resolved by electron microscopy should contribute to the precise interpretation of intracellular deposits of labeled antibody.

In the present context, the surface investigations of Coman and Anderson⁸² provoke speculation. Chromium-carbon replicas of the outer membranes of epidermoid cancer cells (the V2 rabbit carcinoma) reveal a much rougher ultrastructure than is found in corresponding preparations from normal epithelium. The concept of "antigenic displacement" seems in harmony with these electron microscope pictures. The short-range forces that are operative in the formation of complementary antibody molecules against antigenic templates are, according to Pauling,⁸³ effective only over a distance of a few angstroms. The more deep-seated, crowded, or irregularly spaced isoantigens on the coarse membrane of the cancer cell would, therefore, be less efficient templates for stable antibody coating. The V2 carcinoma happens to be a very aneuploid nonspecific neoplasm with a chromosome mode in the triploid region.⁶⁶ Strain-specific diploid tumor cells have normal isoantigenic properties; hence they may resemble normal cells also in their surface morphology. Further comparisons along these lines between diploid and polyploid branches of the same tumor would be instructive.

The molecular pattern of the cell membrane is, no doubt, regulated by H factors and numerous other genes which control the configurations of and the active transport across the living glycolipoprotein lattice. Over-all genic imbalance consequent to heteroploidy thereby has an unavoidable effect on antigenic spacing and type.

Conclusions

Isoantigenic surface properties of mouse tumor cells are controlled by histocompatibility genes that approach the cattle blood groups in their allelic

and pseudoallelic complexity. Immunogenetic characterization of the ascites tumor cells makes it possible to detect genetic changes on the somatic level. These changes may then be correlated with over-all karyotypic balance, though not with events at individual H loci.

All the transplantable tumors capable of growth across isoantigenic barriers have structurally rearranged heteroploid chromosome complements. Tumors with diploid stem lines tend to be strain specific and to regress in foreign hosts. Serologic evidence supports the weakening or disappearance of isoantigens in most cells with irregular sets of chromosomes. Heterologous hemagglutination tests also show a breakdown of species-specific antigens in certain anaplastic polyploids.

Finally, it should be emphasized that "antigenic simplification" of neoplasms is not an absolute phenomenon, but has a meaning only in reference to the test system employed; namely, homotransplantability, or the surface of the normal mouse red cell.

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DISCUSSION: PART I

Wendell Stanley, *Chairman*
University of California, Berkeley, Calif.

VAN R. POTTER (*University of Wisconsin School of Medicine, Madison, Wis.*): I should like to direct a question to Arthur Pollister as to whether or not basophilia in cells should be associated with the ion protein granules that are attached to the endoplasmic reticulum, or whether it does not seem likely that they are to be associated with the particles, or the frequency of particles that are unattached?

ARTHUR POLLISTER (*Columbia University, New York, N. Y.*): The reason for my statement is that you do not find mitochondria staining with basic dye under conditions where we know the basic dyes are bound by nucleic acid. Furthermore, the mitochondrial masses that occur in spermatids are sometimes as much as $20\ \mu$ in diameter and perfectly normal. These are the most ultra-violet-transparent objects in the sperm cell. In ultraviolet pictures the mitochondria often are transparent to ultraviolet radiation indicating at least a very low concentration of ribonucleic acid (RNA). I know of no exceptions to the general rule that these basophilic regions that can be shown specifically as binding R-RNA are the sites of these granules. That is as far as we have been able to go. G. E. Palade may be able to answer further.

G. E. PALADE (*The Rockefeller Institute, New York, N. Y.*): We do not have quantitative evidence bearing on the question asked by Van Potter, but the general impression we have is that the basophilia of the cytoplasm is conditioned by its total population of particles, whether attached or unattached. The number of free particles can be very large in certain cells, and the number of attached particles can be small, or vice versa. In any case, the basophilia is intense when the total population of particles is high.

WENDELL STANLEY: I think that the characterization of these particles will certainly provide many of us with more problems than we can face in the days to come because, if in fact they do represent the enzyme-forming systems and do in fact carry information, then all of the information of the cell presumably must be carried in these particles. In order to separate them out you will have to return to the procedure of Joseph Beard; that is, tie a physical particle to a particular biological activity. In view of what has been said, the latter could be a genetic type of activity.

RENATO DULBECCO (*California Institute of Technology, Pasadena, Calif.*): In reference to a similar question, Joseph Beard referred to the purification of virus, and he defined some virus populations as pure. I should like to know how the criterion for purity could be defined. What I mean is this; it is very easy to say that something is not pure if we can eliminate something from it by some procedure, but are we on equally solid ground in saying that something is pure because we cannot eliminate anything from it with the procedures that we have available? This point is important, because in the present and subsequent discussions there will be questions related, for example,

to pure preparations of host, antigen, and enzyme. Consequently, I should like to have some idea of the criteria of purity.

WENDELL STANLEY: I believe that Beard referred to variations in purity as it occurs in nature. In this specific instance he was commenting upon the great variation in the relative amount of virus in certain chicken sera. When he referred to "pure virus" he was using the term in a relative sense.

GIULIO BOSCO (*University of Rome Medical School, Rome, Italy*): In relation to the problem of physical disruption in the analysis of cell components, I shall present some data from my laboratory concerning the action of high-power ultrasonic waves on the bacterial cell.

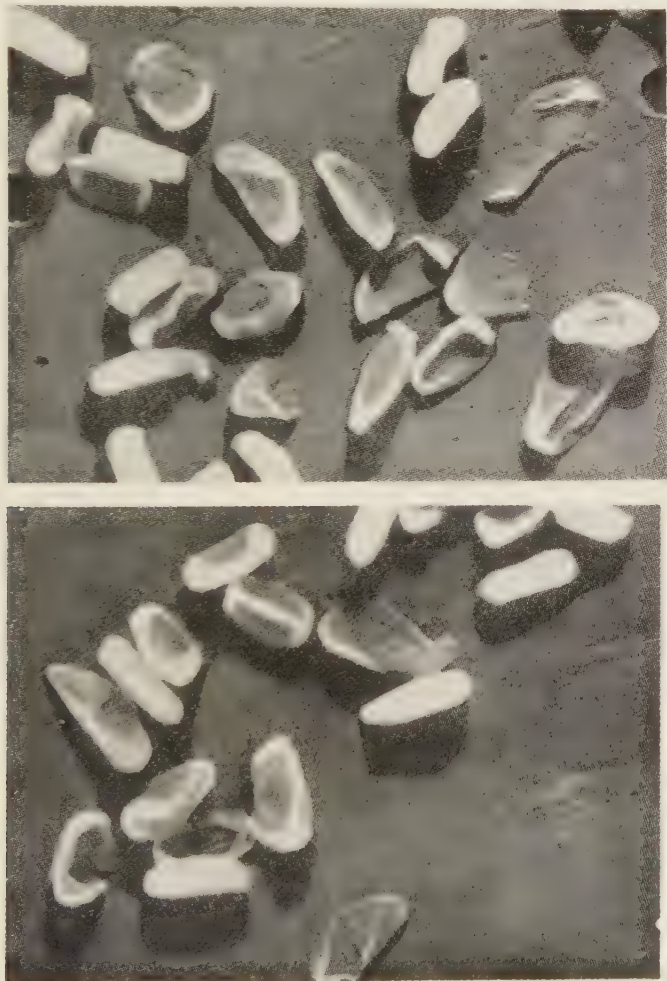


FIGURE 1. The effects of the treatment of cells of *Bacillus mycoides* with ultrasonic waves for 4 minutes. Photographed by means of an electron microscope, with Formvar-coated screens, and shadowed with palladium.

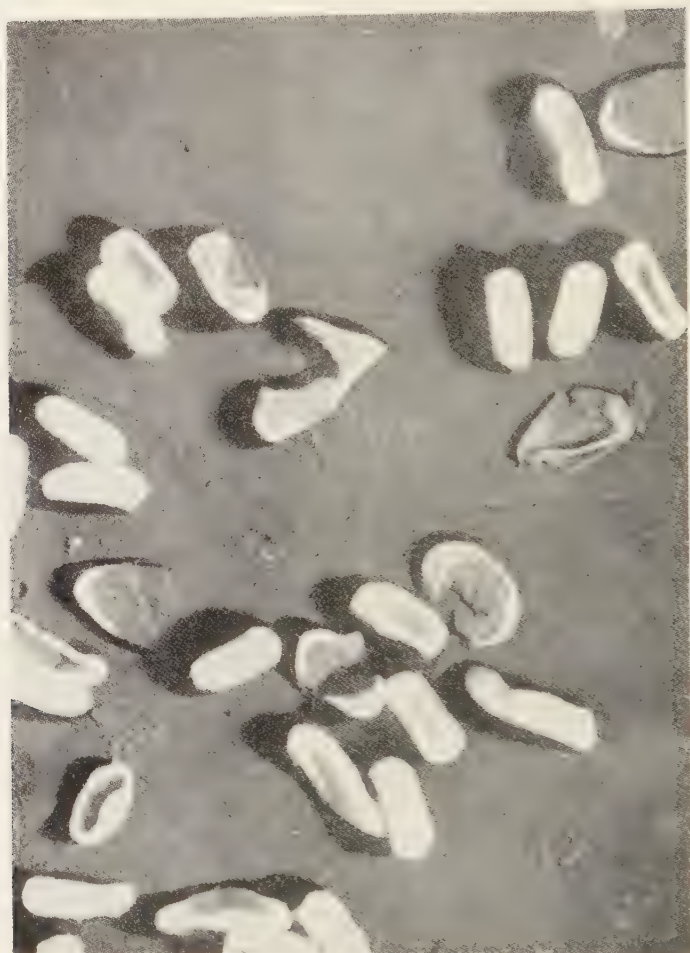


FIGURE 2. The effects of the treatment of cells of *Bacillus mycoides* with ultrasonic waves for 4 minutes. Photographed by means of an electron microscope, with Formvar screens, and shadowed with palladium.

In these experiments ultrasonic waves were used under the following conditions: 800 watts, 2,000 volts, and 400 kilocycles per second.¹

FIGURES 1 and 2² show the results of such treatment on the cells of *Bacillus mycoides* that had been grown for 6 hr. on beef-infusion agar slants. The cell walls appear isolated after a bacillary suspension has been subjected to such treatment for 5 min. (FIGURE 3). This prolonged mechanical shaking is thus able to wash away the last adhering layers of cytoplasm.¹ In addition, it is possible to isolate the chromatinic bodies by means of ultrasonic shaking of cells of *Escherichia coli* in the first hours of incubation (FIGURE 4).³

These preliminary researches show that the use of high-power ultrasonic

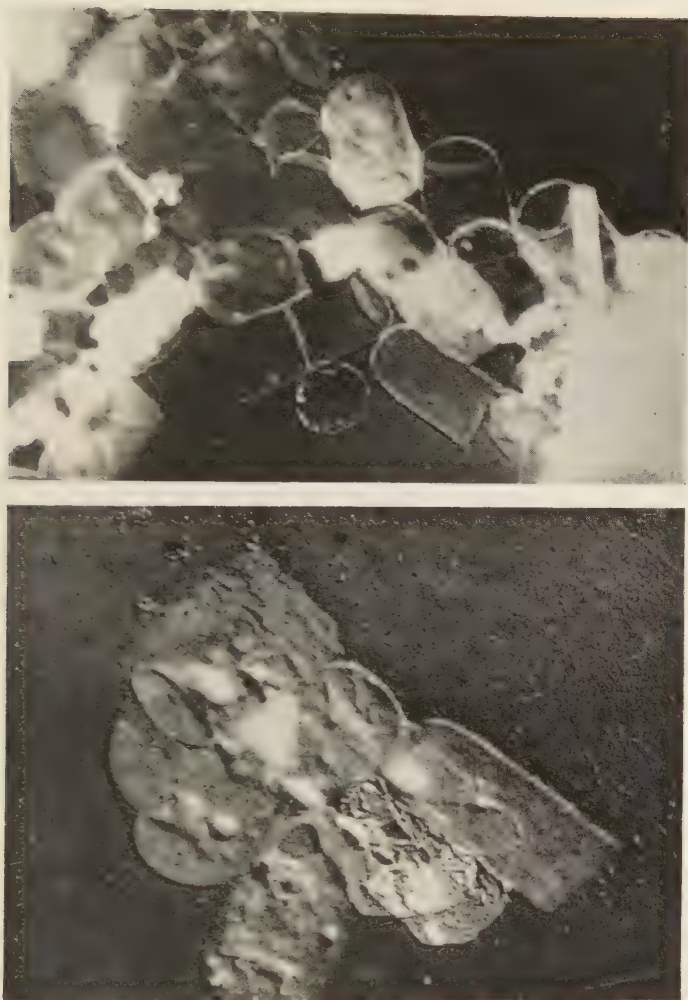


FIGURE 3. The cells shown in FIGURES 1 and 2 after 5 minutes of ultrasonic treatment.

waves can be considered as a simple, rapid, and convenient method for the isolation of cell components.

LESLIE BRENT (*University College, London, England*): If it is indeed accepted that the homologous transplantation antigens reside only in the nuclei of cells, then I find it difficult to envisage a change of cellular antigenicity as a result of changes in the structure of the cell wall. In this connection I wonder whether Theodore Hauschka has ever observed any alterations in the rate of growth of his changed tumors? Could it not be that polyploidy may bring with it an increase in the rate of growth, thus enabling the tumor to sweep aside the immunological opposition of the host? To judge from our work on

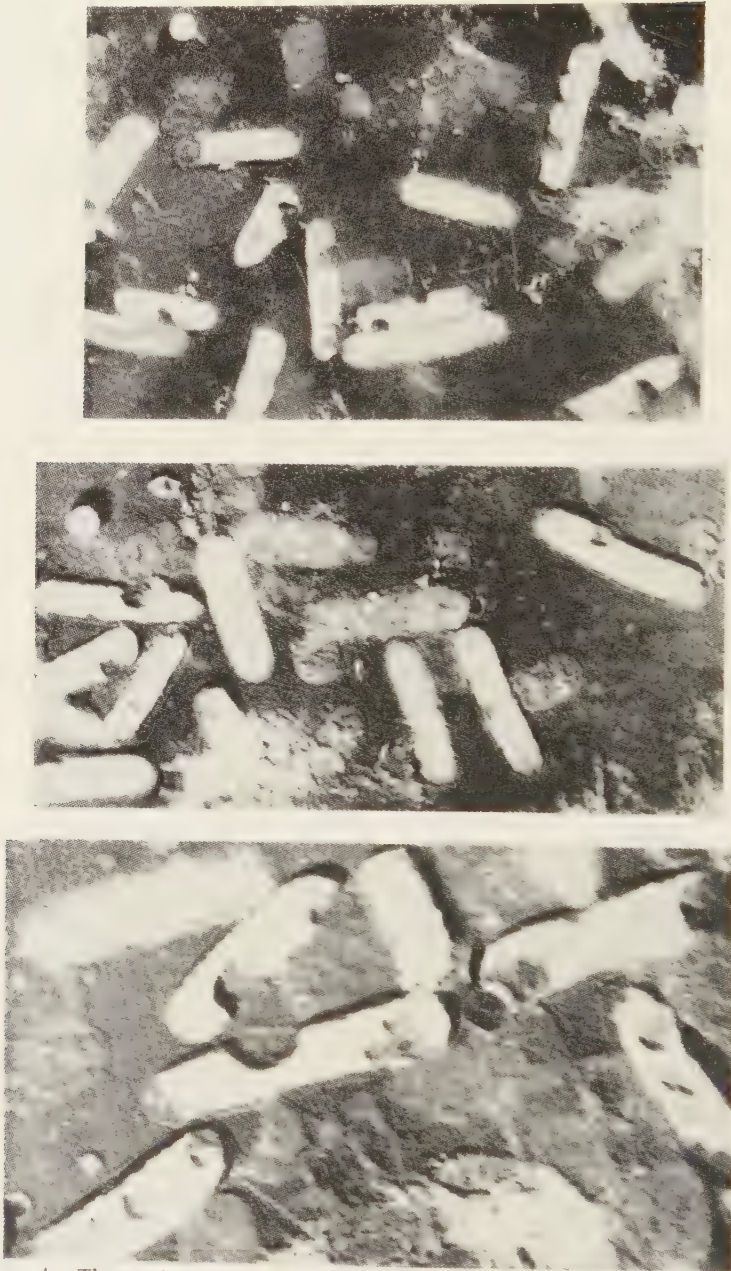


FIGURE 4. The results of ultrasonic treatment of cells of *Escherichia coli*, showing the isolation of the chromatinic bodies.

acquired tolerance, it is certainly possible to state that certain tumors can grow progressively in the face of weak immunity in the recipients.

THEODORE H. HAUSCHKA (*Roswell Park Memorial Institute, Buffalo, N. Y.*): We have compared the rates of growth of a hyperdiploid Ehrlich ascites and its tetraploid subline. These two lines are genetically comparable, since the latter line was derived from the former by immunoselection, and both of them have been propagated in the same stock of mice. To our surprise, we have found that the rate of growth for the two cell populations is exactly the same until a limiting mass is reached. This occurs sooner for the tetraploid subline because of the larger individual volumes of its cells. The lethal mass of tumor protoplasm per mouse that is synthesized by the tetraploid and the diploid is nearly the same, but the total number of cells per mouse is roughly twice as large for the hyperdiploid population, which is in keeping with the smaller individual cell volumes. This is interesting from the point of view of mass regulation by a host strain. Our observation has been confirmed by Klein and his collaborators at the Karolinska Institute, Stockholm, Sweden. These investigators, in their extensive growth data for various diploid and polyploid tumors, have found no correlation whatever between the rate of growth and histocompatibility.

ROBERT R. CITRON (*Stockton, Calif.*): My comments are directed to Jerome Syverton, Van Potter, and Theodore Hauschka. I should like to quote Syverton's remarks anent the myriad of mixed antigens in cancer cells and Potter's observation that the function of lipoprotein is in urgent need of greater study. Hauschka's very interesting microphotographs dealing with the irregularities of surface between normal cells and cancer cells may also lend support to the apparent significance of lipid and lipoprotein in this area of medical research. All of the foregoing makes me wish to call attention to some work in this field that has been done for a number of years concerning cancer lipid as antigen; that is, as an antigenic fraction derived from cancer tissue by means of fat solvent as, for example, by ethyl ether extraction techniques. This was reported recently at the meeting of the American Association for the Advancement of Science in New York, N. Y. I wish to emphasize this approach as a means of correlating the various ramifications that point to some common denominator present in cancer tissues that has specific antigenic activity, in contrast to components of normal tissue that have not. It does not appear that protein alone holds the solution to this problem, and I mention the concept of lipids and experimentation with them as means of throwing light on the controversial situation that has existed heretofore in this difficult field of cancer immunology.

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Part II. Immunological Techniques and Their Applications

THE PRECIPITIN REACTION

By Otto J. Plescia

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The current emphasis in immunological studies of cancer has been on the basic problems of tumor specificity and the chemical nature of cellular antigens. In the broad sense, the principal problem pertains to the relationships between host and tumor. In host response to a foreign substance, antibodies are generally found in the circulation. This is usually regarded as the result of an immunological response, but it is only one manifestation of host defense. Because a number of basically different responses, each a part of the over-all defense mechanism, are possible in host-tumor relationships, it is operationally expedient to define immunological response as one in which specific antibodies are produced, even though it is not certain that these antibodies have a direct bearing on host resistance. It is in the estimation of antibody response that immunochemistry can and does play an important part in cancer research. Moreover, because of the precise relationship between the chemical constitution of an antigen and its immunological specificity, immunochemistry should also play a decisive role in the study of tumor-specific antigens, if any.

Among those reactions that form the basis of immunochemistry and are applicable to problems in cancer research is the precipitin reaction, used to measure precipitating antibodies. Ever since the discovery of this reaction by Kraus¹ in 1897, it has been studied and used extensively. Its widespread use stems from the fact that it is a precise analytical reaction that rests upon the quantitative method developed about twenty-five years ago by Heidelberger and Kendall.² In the study of tumor specificity it is especially important to use strictly quantitative methods because, as will be discussed shortly, cross reactions are possible between a given antibody and immunologically related antigens.

The reaction of antiserum with a solution of antigen often results in the formation of an immune specific precipitate that can be washed free of the nonspecific constituents also present. The amount of antibody in the specific precipitate can be determined quantitatively in weight units by the rigorous methods of analytical chemistry. The relationship between the quantity of antibody precipitated and the antigen added is characterized by three distinct zones; that is, a region of antibody excess; an equivalence zone in which neither antigen nor antibody can be detected in the supernate after precipitation; and a region of antigen excess. The amount of antibody precipitated at equivalence or slight antigen excess is a measure of the content of precipitin in the serum. The general shape of the precipitin curve is of interest because it varies, depending upon a number of factors, notably the specificity of the reaction and the number of distinct antigen-antibody systems present; both of these factors are important in the use of the precipitin reaction in the study of tumor antigens. When a mixture of reactive antigens is used, precipitation generally

diminishes less rapidly and extends farther into the region of antigen excess. This characteristic, however, is not restricted to mixtures of antigens only. The antitoxin-flocculation reaction differs from the usual precipitin reaction in that there is an initial zone in the region of antibody excess in which no precipitation occurs. This is a property of the antibodies produced by certain antigens in the horse, for the same antigens stimulate production of precipitins in other animals.

The most important single aspect of the precipitin reaction is its specificity. Despite differing views as to the nature of the forces and mechanism of interaction between antigen and antibody,⁹⁻¹⁰ a chemical basis for immunochemical specificity is becoming firmly established. The early work of Landsteiner¹¹ showed that a protein coupled with a simple substance (hapten), such as *m*-aminobenzenesulfonic acid, could elicit antibodies directed specifically to the hapten. Not all the antibodies were specific for the hapten; a portion of them reacted with the protein alone and others reacted with the protein-hapten complex. It appeared, therefore, that the antigen molecule contained a number of determinant chemical groups, each contributing to its immunological specificity. Aside from the hapten, little could be said concerning the chemical nature of the naturally occurring determinant groups of the protein molecule itself. The fact that simple substances could stimulate the production of specific antibody influenced subsequent thinking and research. This observation, for example, led Creech¹² and his associates to study the immunological properties of chemical carcinogens coupled to proteins as haptens.

The specificity of the precipitin reaction may be revealed by a study of either cross reactions or of inhibition reactions with immunologically related antigens. A typical cross reaction between protein antigens is the precipitation of rabbit anti-chicken egg albumin (Ea_c) by duck egg albumin (Ea_d), studied quantitatively by Hooker and Boyd¹³⁻¹⁵ and also by Osler and Heidelberger.¹⁶ The precipitin curves for both the homologous reaction and the cross reaction are shown in FIGURE 1. It is to be noted that both Ea_c and Ea_d precipitate antibody and that the two curves are generally alike in appearance. The striking and significant difference is between the quantities of antibody precipitated; the cross-reacting antigen could precipitate only a portion of the antibodies capable of reacting with the homologous antigen. Apparently the molecules of antibody against a given single antigen are not immunologically identical; among them there are some that can react with chemically related substances to extents depending upon their degree of similarity with respect to immunological determinant groups. Therefore, in attempting to identify antigens by means of the precipitin reaction it is not sufficient to know only that they react qualitatively with the same antiserum. One must also know the extent of reaction on a quantitative basis over much of the range of the precipitin curve. For the case of single pure immunologically identical antigens, their precipitin curves should be superimposable.

The elucidation of the fine structure of proteins is slow and laborious. However, the fine structures of many polysaccharides have been worked out, and Heidelberger¹⁷ has been studying extensively the immunological specificity of this group of substances. Already his results have extended the concept

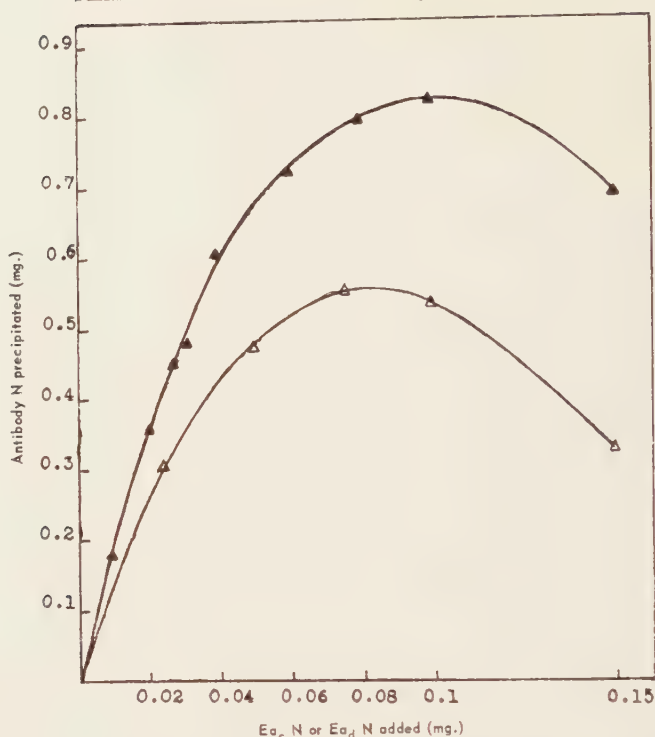


FIGURE 1. Precipitation of antibody N at 0° C. from rabbit and anti-Ea_c serum 791 by increasing quantities of Ea_cN (solid triangles) and Ea_dN (open triangles). Ea_c = chicken egg albumin; Ea_d = duck egg albumin. Reproduced by permission from *The Journal of Immunology*.¹⁶

of a chemical basis for immunological specificity. For example, he has separated the immunological specificity of Type II pneumococcus into three partial specificities, each characteristic of one of the three component sugars of the Type II pneumococcal specific polysaccharide, D-glucuronic acid, D-glucose, and L-rhamnose. Quantitative measurements were made on cross reactions of Type II pneumococcus antiserum with polysaccharides known to contain at least one of the component sugars of the Type II pneumococcus polysaccharide. The extent of cross reaction depended, not only upon the presence of at least one component sugar or sugar acid, but also upon their distribution and linkage in the molecule as a whole. Since the antibodies in the serum were directed against multiple recurrences of a rhamnoglucoglucuronic acid, the sum of the partial specificities measured was not equal to the whole specificity, owing undoubtedly to the poorer fit of the antibodies with cross-reacting substances. For this reason, precipitation of antibody by a cross-reacting substance is generally quite sensitive to temperature, so that the reaction is best carried out at 0° C., and greater amounts of antigen and longer reaction periods are usually required to attain maximum precipitation.

As another instance of the specificity of the precipitin reaction, I shall cite

one that demonstrates the power of immunochemistry in the study of chemical composition. Heidelberger¹⁸ found that lung galactan, isolated by Wolfrom *et al.*,¹⁹ precipitated antibody from Type XIV antipneumococcus serum owing to the presence of galactose in both. Unexpectedly, the lung galactan also precipitated Type II antiserum. It developed that there was present an unknown uronic acid to the extent of one residue for every 35 to 40 galactose units. Precipitation with Type II antiserum could be explained, then, provided that the unknown uronic acid was glucuronic acid. With this clue Wolfrom and Neely¹⁸ later did identify it as glucuronic acid. Also, the carbohydrate in the immune precipitate with Type II antiserum was analyzed and was found to be richer in glucuronic acid than the carbohydrate from the precipitate with Type XIV antiserum, showing the galactan to be inhomogeneous.

In our laboratory an attempt is being made to isolate and characterize the mammary tumor agent from milk of mice. For this a rapid and precise method of assay for the agent is needed because of the extreme time, at least one year, required for bioassay. In view of the apparent antigenicity of the agent in heterologous hosts,²⁰ we are attempting to develop an immunochemical method based upon the precipitin reaction. Rabbits have been immunized with defatted milk of the R III strain of mice known to contain the milk agent. Because a mixture of antigens was used for immunization, the antiserum produced in this way contains several antibodies, most of them undoubtedly directed against normal milk antigens. In order to eliminate those antibodies to normal constituents of milk, the antiserum ideally should be absorbed with milk from an isogenic mouse strain free of the factor; because none is available, it is being exhaustively absorbed with serum from the C57 strain of mice free of the milk agent. The antiserum will be absorbed next with extracts of mammary tissue from C57 mice and then examined for precipitins against factor-containing R III mouse milk. It is hoped that any precipitins remaining will be specific for the tumor agent and will not cross-react with other milk constituents. Unfortunately, analysis for such agent-specific precipitins depends at first on bioassay. However, once precipitins against the milk factor have been demonstrated, the precipitin reaction can be set up quantitatively and used to follow the tumor agent in the fractionation of milk. Already R III milk has been separated into its electrophoretic components by means of zone electrophoresis on a column of powdered cellulose, but further purification is not possible unless the fraction containing the factor is first established. Agent-specific precipitins, in addition to providing a rapid method of assay, may possibly be used to isolate specifically the tumor agent for characterization studies.

In conclusion, the precipitin reaction may afford a precise tool for establishing the specificity of tumor antigens, if any exist and can engender precipitins in some heterologous host, provided it is carried out on a strict quantitative basis and provided also that due consideration is given to the possibility of cross reactions. Even when necessary precautions are taken, the precipitin reaction is limited in its usefulness for the study of tumor specificity. Because of the lack of suitable methods for the isolation of single, pure tissue

antigens, immunization is ordinarily carried out with a mixture of antigens, and antibodies to the several antigens are therefore probably present in immune sera obtained in this way. In such mixtures it is generally difficult to separate each antigen-antibody reaction for individual study. In some instances this problem can be resolved by carrying out the precipitin reaction in a gel and allowing the antigens and antibodies to diffuse toward each other. Pierre Grabar discusses this technique in detail elsewhere in this monograph.

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AGAR-GEL DIFFUSION AND IMMUNOELECTROPHORETIC ANALYSIS

By Pierre Grabar

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The methods that I have been invited to present here are based on the specific precipitin reaction, the principles of which have been described elsewhere in these pages by Otto Plescia.

Since 1905² several authors have used gelled media in order to stabilize and facilitate the observation of the interfacial precipitin test, or "ring test." It is interesting to recall the work of Nicolle, Césari, and Debains³⁴ who, in 1920, used such a medium (gelatin) in order to avoid the rapid dissolution of the specific precipitate in the presence of excess antigen. They observed the formation of a precipitation band in the gel and proved that this precipitate was specific. They have also used this technique to show the presence of antihorse serum antibodies in the sera of persons treated with antidiphtheria horse serum. This method was adapted mainly for the titration of toxins using serial dilutions. These results have been confirmed and extended by Hanks.²³

The lack of knowledge, at that time, of the precise mechanism of the specific precipitation reaction probably explains why this method was not developed further. On the other hand, some investigators observed the formation of several lines or bands of precipitation and, under the influence of colloidal chemistry, they wondered if at least some of these bands could not be due to the formation of Liesegang rings.⁶⁻⁴⁹ Oudin³⁷ has described the simple diffusion method and has shown that the number of precipitation systems present in the studied solution is equal to or greater than the number of observed lines. In this method, tubes are used and, generally, the antigens (used in excess) diffuse into an agar gel that occupies the bottom of the tube and contains the immune serum.

Working independently, Ouchterlony³⁶ has developed a double-diffusion method in which a flat agar gel formed in Petri dishes is used. The antigen solution and the immune serum are disposed in troughs made in the gel. In a publication that appeared at the same time, Elek¹² described an analogous technique, but on the agar gel he placed paper strips soaked with the reagents. In these techniques both the antigens and the antibodies diffuse into the gel, which originally contains neither antigens nor antibodies and, when they meet in suitable proportions, a visible line or band of precipitate is formed.

More recently numerous modifications or variants of these methods have been suggested. In most cases they concern the forms or the relative positions of the troughs in the agar plates, or the use of tubes for double diffusion.^{35, 50}

In all methods using gels the formation of these bands is conditioned by the physical properties (that is, the diffusion coefficients) of both antigens and antibodies and by the respective quantities of these reactants, the precipitation of which is governed by the quantitative relations established twenty years ago by Heidelberger and his school.

The general laws of diffusion in gels were established long ago by physical chemists using simple, mainly inorganic, substances.^{43, 53} The same laws seem to apply in the case of immunochemical precipitating systems,^{3, 36, 37, 45} and it is even possible to reproduce the characteristic patterns of Ouchterlony with mineral substances.^{43, 44} The rate of diffusion of some proteins in agar gel has been measured, using their reaction with the homologous antibody.^{3, 36, 60} The results have been used to establish a method for the quantitative determination of antigens,^{3, 42} but some difficulties arise because it seems that other constituents may interfere.³⁵ Therefore, at the present time, there is no absolute quantitative method for the estimation in gelled media of antigens or antibodies in mixtures. However, it is possible to make relative quantitative estimations using serial dilutions. Thus, Augustin¹ has described such a method derived from the double-diffusion technique in tubes of Oakley and Fulthorpe,³⁵ and has used this method for the relative quantitative determination of the concentration of allergens in preparations of different purity.^{14, 60} At the present time, the gel-diffusion methods are mainly used for qualitative analysis, and they are yielding very useful information.

The techniques that utilize the simple diffusion in tubes allow one to count the minimum number of constituents in a mixture. By using sufficiently high concentrations of the antigens, one obtains better separation of the different lines of specific precipitation.

The use of double-diffusion agar plates has the advantage of allowing one not only to determine the number of precipitating systems, but also to compare different antigenic solutions or immunesera on the same plate. As has been established by Ouchterlony^{31, 36} three patterns can be foreseen, corresponding to three different cases: identity of antigens, cross reaction, and different antigens (FIGURE 1). However, a pattern resembling a cross reaction can be observed even with the same antigen, if highly different concentrations of it are used.^{25, 27, 32}

In the case in which several lines appear, it is possible to identify at least some of them by the use of solutions of pure substances or different preparations. If the line given by a pure substance is confluent with one of the lines formed by the mixture, it can be concluded that this line corresponds to that substance. The same principle can be applied in order to demonstrate the presence of the same component in different mixtures (FIGURE 2). The preliminary absorption of one of the antibodies by a pure antigen also may be used for the identification of one of the lines.

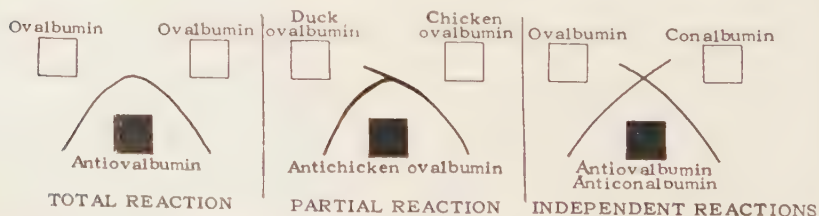


FIGURE 1. The principle of Ouchterlony's double-diffusion method.³¹ Reproduced by permission from the *Bulletin de la société de chimie biologique*.

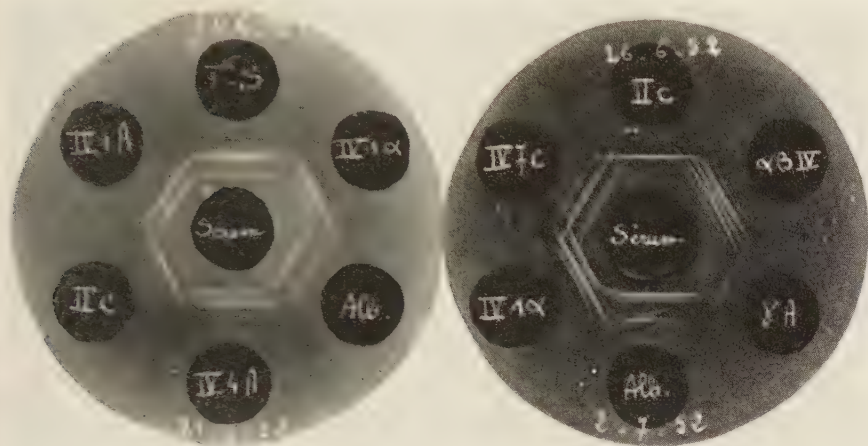


FIGURE 2. The analysis of different fractions of human serum by means of horse-immune serum (center) by Ouchterlony's method.^{20a}

If the antigen-antibody ratio is near to the equivalence point, the precipitate forms a fine line; if not, it forms a band that possesses a relatively sharp edge on the side of the reagent (antigen or antibody) which is not in excess, and a turbid zone on the other side. This is practically always the case in the simple-diffusion method, in which an excess of antigen is generally used. Several turbid zones may overlap and make interpretation difficult when a complicated mixture is examined.

As already mentioned, various dispositions or forms of the troughs have been described. Some of them are mathematically ingenious as, for example, the triangle system of Jennings²⁶ (FIGURE 3). In the cases in which many different solutions are to be compared for their antigenic components, we use gels formed on photographic plates as described by Kaminski.²⁹ The troughs are disposed in a "chessboard" pattern (FIGURE 4) or in parallel lines. This last system is particularly suitable for testing the supernates when precipitation curves are established (FIGURE 5), and for counting the components in antigenic solutions. It permits one to determine the quantity of antigenic solution necessary for the absorption of one or several antibodies from the immune serum, or to ascertain which of the antibodies is absorbed when a certain amount of the antigen mixture has been added to an antiserum (FIGURES 6 and 7).

Bjorklund⁴ has proposed using histochemical reagents in order to stain the specific precipitation lines in the gel. This procedure allows one to identify some antigens, such as lipoproteins, in a mixture.

The large number of published applications of these methods prove their usefulness.* They can be helpful in many very different researches: for

* Such applications can be found, for example, in Augustin and Hayward,¹ Becker *et al.*,³ Burtin *et al.*,⁹ Elek,¹² Fauvert *et al.*,¹³ Feinberg,¹¹ Grabar,¹⁷ Grabar and Lapresle,^{20a} Jennings and Malone,²⁶ Kaminski,²⁹ Kaminski and Durieux,³⁰ Korngold,³² Oakley and Fulthorpe,³⁶ Ouchterlony,³⁶ Oudin,³⁷ Relyveld *et al.*,³¹ Staub and Pon,³⁹ Seligmann, Grabar, and Bernard,⁶² Telfer,⁶⁴ and Wodehouse.⁶⁰

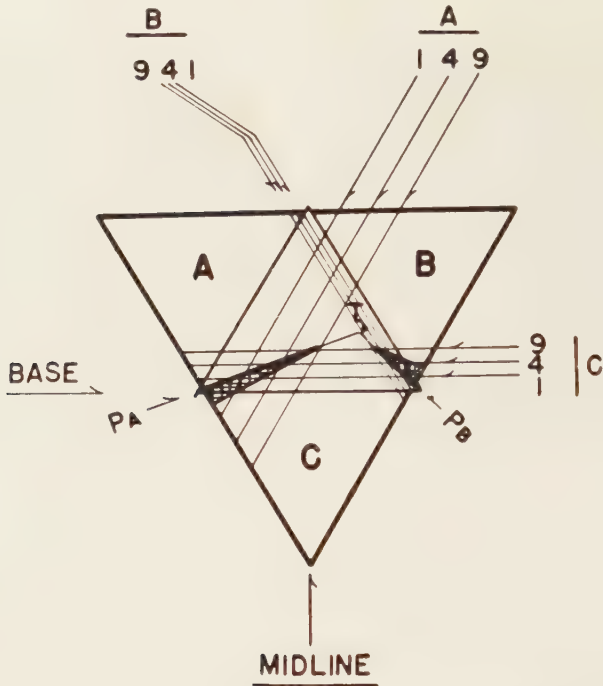


FIGURE 3. The principle of Jennings' double-diffusion technique, shown in a diagrammatic representation of diffusion and zone formation. Areas A, B, and C are reagent depots. The figures 1, 4, and 9 are isobars of arbitrarily chosen reagent concentration as located at 1-, 4-, and 9-hour observation times, respectively. P_A and P_B are the paths of precipitate formation. T is the point of theoretical coalescence of zones of precipitate. Reproduced by permission from *The Journal of Immunology*.

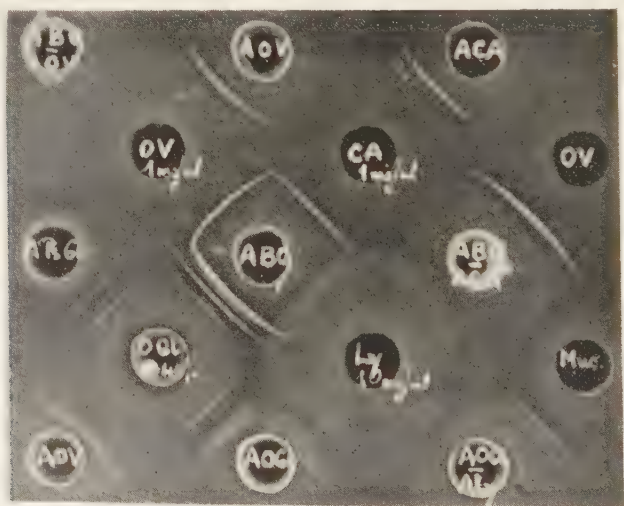


FIGURE 4. Chessboard pattern of troughs for double diffusion.

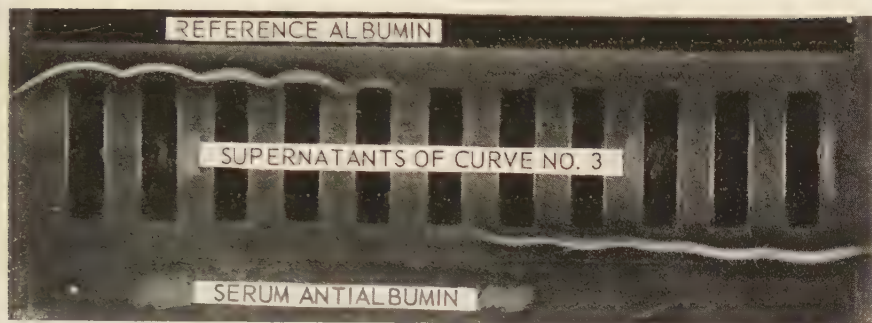


FIGURE 5. The control of supernatants of a specific precipitation curve (human serum albumin plus rabbit antiserum).³³

controlling the purity of different preparations, for comparing or establishing the composition of natural mixtures, and so on.

However, these methods also have inconveniences, which are of three orders:

(1) As mentioned above, the position of the lines of precipitation depend on the diffusion coefficient and the antigen antibody ratio. It happens relatively often that bands are superposed. In order to control this difficulty one can change the proportions of the reactants or utilize several immune sera obtained from different animals, because it is probable that they contain varying amounts of antibodies for the different antigens of the mixture.

(2) Although in principle the number of lines must correspond to the minimum number of independent precipitating systems in the studied mixture, it has been shown that a single precipitating system may give more than one line. This phenomenon is frequently seen in Ouchterlony plates at the angles of junction of two lines.⁷ A systematic study, made by Kaminski^{28, 45} has demonstrated that, in the presence of an excess of one of the reactants, the bands enlarge and, if the excess is very great, these bands dissociate in several lines or bands, the number of which increases with time (FIGURES 8 and 9). Analogous observations seem to have been made also by Jennings.²⁵ Two explanations of this phenomenon can be envisaged: the multiplication of bands could be due to a mechanism similar to the formation of Liesegang rings. On the other hand, we know that antibodies in an immune serum are heterogeneous; they can be more or less of the precipitating and nonprecipitating types, and there are antibodies reacting with different "antigenic motives" on the antigen molecule. Because of this heterogeneity the maximum precipitation points may not coincide. We think that at the present time a correct explanation of this phenomenon cannot be given, and that further experimentation is needed. However, the possibility of multiple bands given by a single antigen must not be overlooked.

Another case of the appearance of several lines corresponding originally to one antigen has been observed and explained by Lapresle.³³ This investigator has shown that human serum albumin (HSA), when submitted to the action of an endocellular protease (the optimum pH is 3 to 3.5), is first broken in degradation products, which give, in double diffusion, three independent lines

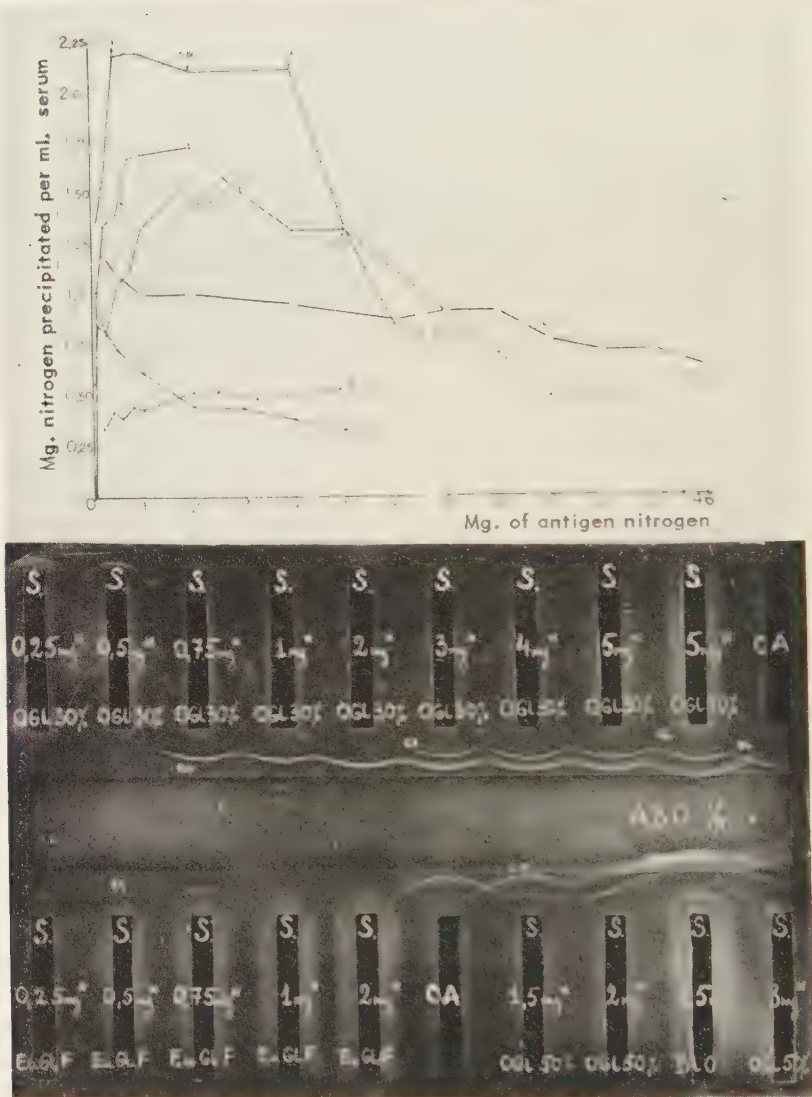


FIGURE 6. Specific precipitation curves and control of supernatants in the case of a mixture of several precipitating systems (egg white and its fractions plus rabbit anti-egg-white serum).²⁹

with the anti-HSA rabbit serum. This serum thus contains at least three different antibodies that react with antigenic motives situated in different parts of the original native serum-albumin molecule. From this observation it must be concluded that, if an antigenic molecule is cleaved during its isolation or purification, one can observe several precipitin lines. Such a case has been

observed in studies on different purified gamma-globulin preparations. Some of them gave two lines, which coalesce with the single line given by other preparations (FIGURE 10).¹⁸

(3) The third difficulty that can be encountered in the gel-diffusion techniques is the definition or identification of the constituents of a mixture of antigens. As I have said before, it is possible, by the Ouchterlony method,

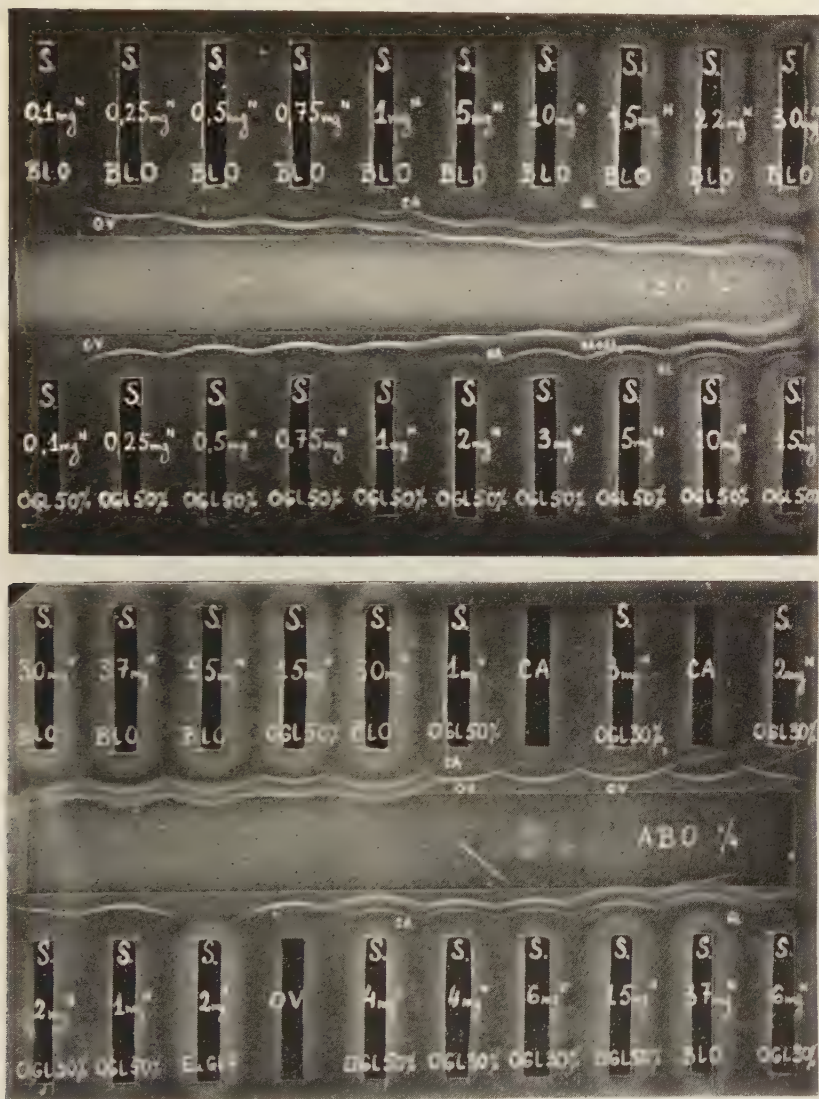


FIGURE 7. Control of supernatants in the case of a mixture of several precipitating systems (egg white and its fractions plus rabbit anti-egg-white serum).²⁹

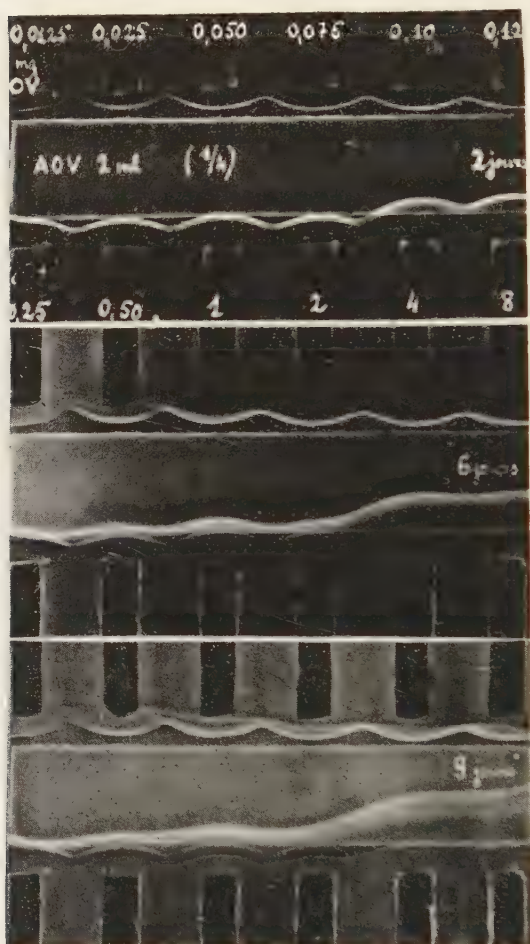


FIGURE 8. Dissociation of specific precipitation bands, as a function of time, with a single antigen-antibody system (ovalbumin plus antiovalbumin rabbit serum) and in the presence of an impurity (conalbumin).²⁸

to identify a line if the corresponding substance is available in a purified state. In many cases, however, particularly in the examination of natural liquids, the number of the constituents is large, and only a few of them have been isolated in pure state. We have encountered this difficulty on several occasions. For example, when we tried to study human serum by the double-diffusion method, only a few of the many observed lines could be identified (FIGURE 2).^{20a}

In our studies with J. Bernard and M. Seligmann of the constituents of human normal and leukemic leukocytes, the double-diffusion method proved to be very useful.⁵⁰ We have used it for controlling the washing of the cells in order to eliminate the serum constituents, as well as to prove that these

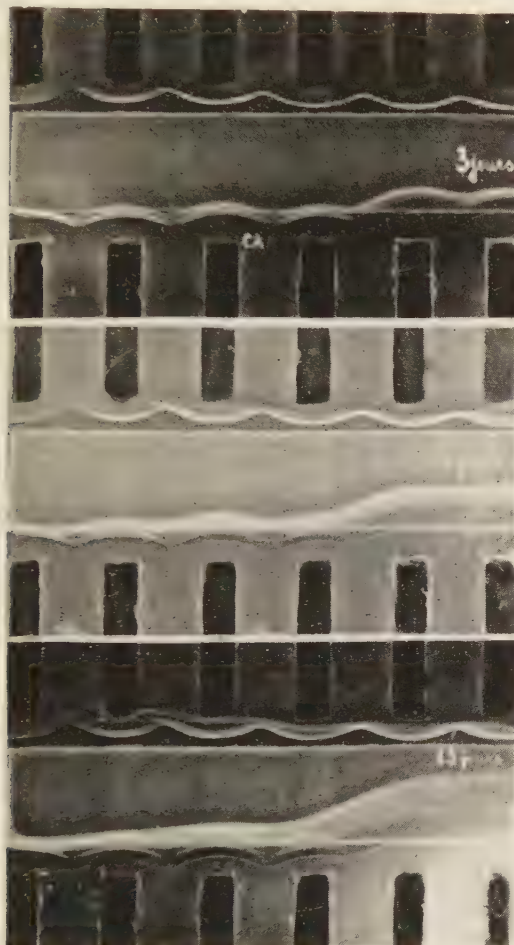


FIGURE 9. Continuation of FIGURE 8.

well-washed cells do not contain the main serum proteins but, instead, at least nine independent leukocytic constituents. Recently it could be proved that at least one of the constituents of the normal leukocytes is present in normal serum in very small amounts, but that larger amounts and, generally, several components of these cells could be detected in the serum of myeloid leukemic patients (FIGURE 11).⁵¹ However, we could not define these substances by this method, and supplementary information had to be obtained by the immunoelectrophoretic analysis.

In order to overcome at least some of the difficulties encountered in the gel-diffusion methods, we have developed, with C. A. Williams, Jr., a method that we call "immunoelectrophoretic analysis."^{52(a), 21b} This is a combination of electrophoresis in a gel with double diffusion, as in Ouchterlony's method.

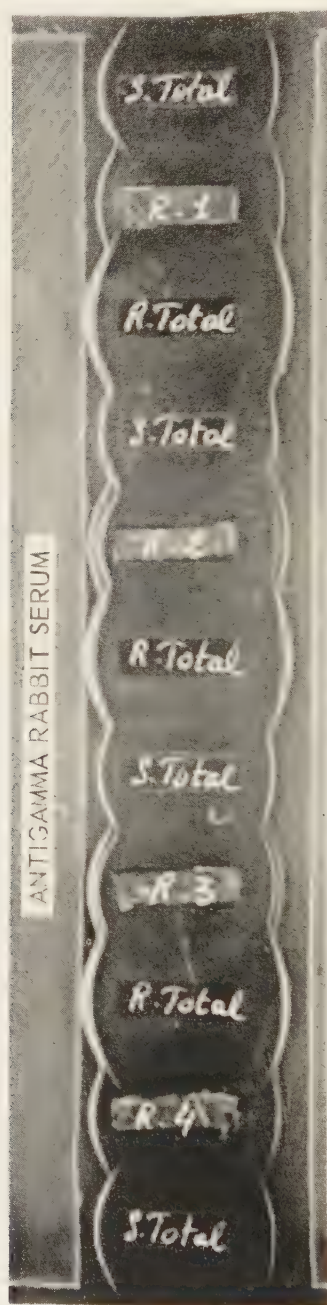


FIGURE 10. The presence of two components in two subfractions (R2 and R3) of certain purified gamma globulin preparations, giving confluent lines with total gamma globulin preparations. Fractions R1 and R4 give partially confluent lines (cross reaction type).¹⁵

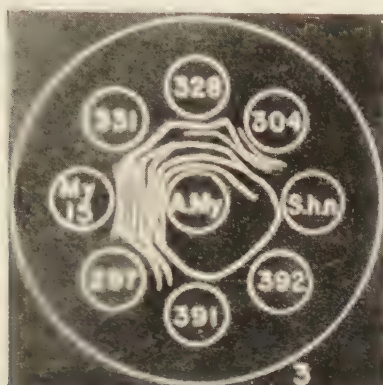


FIGURE 11. Scheme of a double-diffusion pattern given by a rabbit antimyeloid leukocyte serum (AMy) with an extract of myeloid cells (My 15) with sera of chronic myeloid leukemia: untreated (304, 328, 331, 297), in remission (391, 392), and with normal human serum (S.h.n.).⁵¹

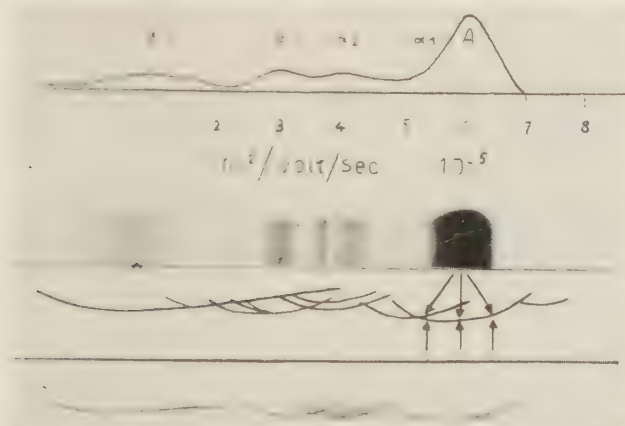


FIGURE 12. The principle of immunoelectrophoretic analysis of human serum compared with (top) the free electrophoresis diagram, (middle) the simple gel electrophoresis with colored spots, and (bottom) a photograph of an immunoelectrophoretic analysis. The arrows show the directions of diffusion of antigen and antibody.²¹

The gel is made on glass plates, and the constituents of the studied mixture are first separated by electrophoresis.* Then the antibodies are allowed to diffuse from a lateral trough and, when they meet the different antigens in convenient proportions, they give specific precipitates in the form of arcs in the gel (FIGURE 12).

The results can be recorded by simple contact photography, and the development of the arcs can be followed during many days. Moreover, a very simple technique of drying the gel allows one to transform it into a thin transparent film in which the lines can be colored by different reagents in order to

* An agar-electrophoresis method in tubes has been described recently by Crowle.¹⁰

furnish information on the chemical nature of some of the antigens. Staining techniques have been described for the characterization of carbohydrates, lipids, acetalphosphatides, proteins, and copper.^{55, 57} Finally, the transparent thin film of agar can be detached from its glass support; this dried film is elastic and can be preserved without special precautions.

A micromodification of our method has been described by Scheidegger;⁴⁶ it may be useful if only small amounts of substances are available and if a rapid result is desirable.

Most of the experiments realized so far have been done with agar gels, but pectin gels can also be used.^{20b} Slater has performed an electrophoresis in starch followed by a diffusion in agar, and his results confirm those obtained directly in agar gels.⁴⁸

As compared to other methods, the immunoelectrophoretic analysis presents the following advantages:

(1) Even very complicated mixtures can be analyzed, and the number of their constituents determined, because the chances of superposed arcs are small. Such a coalescence of arcs can take place only if two constituents have exactly the same electrophoretic mobility and if, moreover, the rate of their diffusion and their antigen antibody ratios are such that the two arcs will be formed at the same place. It seems a priori that a triple coincidence of this kind would have a very low probability.

However, supplementary controls can be performed by changing some of the experimental conditions, such as the use of another immune serum, varying the relative proportions of the analyzed liquid and of the immune serum, changing the distances between the antigens and the antibodies (that is, the distances between the central trough in which the analyzed liquid is placed and the lateral troughs that contain the immune serum).^{17e, 17f}

(2) In this method it is easy to distinguish two neighboring arcs from the possible formation of several bands due to a single antigen, which is provoked, as mentioned before, by a large excess of one of the reactants. Their appearance and evolution can be followed and they can be recognized because of their characteristic forms.^{17e, 17f}

(3) The constituents of a mixture can be defined by their electrophoretic mobilities. Thus, even those substances that have not yet been obtained in pure states can be classified on the basis of this physicochemical property.²¹

It is my opinion that the calculation of *absolute mobilities* in a medium that is arbitrarily chosen (concentration and nature of the gel, ionic strength, and the chemical nature of the buffer solution) is not of particular interest. Therefore, we prefer to characterize the substances by their *relative mobilities* and to compare these mobilities with those of well-defined substances that have been correctly measured in the Tiselius apparatus. The determination of relative mobilities with the immunoelectrophoretic method can be based on the fact that the top of the precipitin arc (that is, the more distant point of the arc from the axis of migration) corresponds to the point at the end of migration at which the analyzed substance was at the highest concentration. This point can be determined in this manner.¹⁸⁻³⁹ Since the agar possesses a certain electrical charge, there is, during electrophoresis, also an electroendosmotic

flow of the liquid in the opposite direction. However, the relative distribution of the different constituents of the examined fluid seems not to be affected by this electroendosmotic flow.

Among the different substances studied so far, only one, the lysozyme of egg white,²⁰ has been retained by the agar gel. For all the other substances, the mobilities of which have been determined in the Tiselius apparatus, the relative migrations in the agar gel have been found to be analogous.⁵ If compared to the results obtained by paper or starch electrophoresis, however, there is one serious discrepancy, namely, the lipoproteins of the serum. On paper, the principal lipoprotein migrates as a beta-globulin, whereas in agar it has the mobility of an alpha-globulin, and the two other lipoproteins have the mobilities of the albumin and of the ρ fraction.⁵⁶

Immunoelectrophoretic analysis is extremely simple and does not necessitate expensive installations (FIGURE 13). The main difficulty lies in obtaining potent precipitating antisera containing antibodies to all of the antigenic constituents of the studied mixture. This inconvenience is general for all the methods based on immunochemical reactions, and the variations of the response of animals to the injection of a mixture of antigens are universally

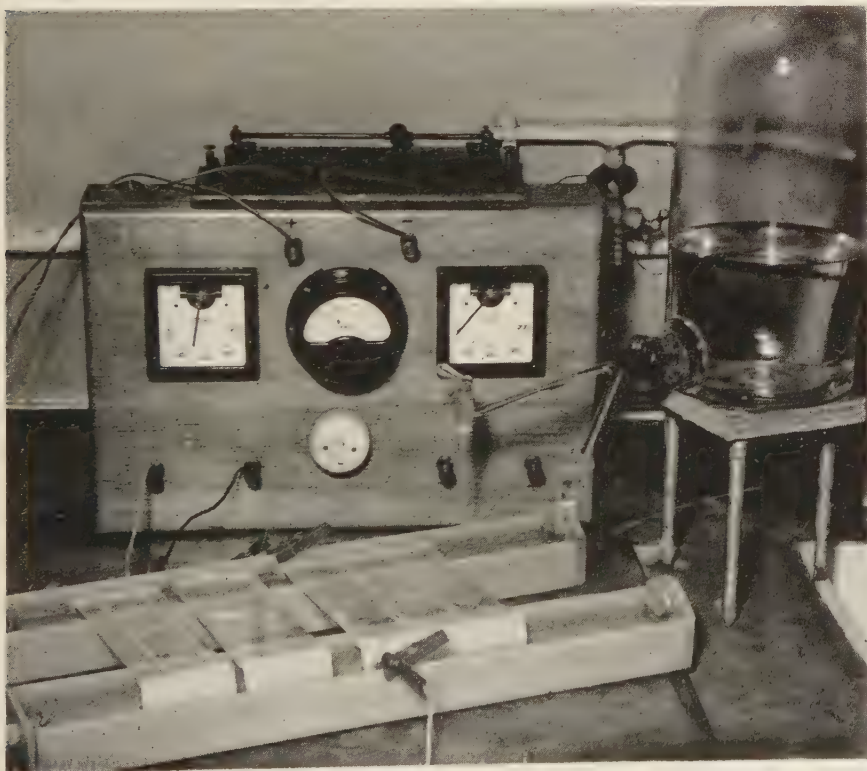


FIGURE 13. Apparatus for immunoelectrophoretic analysis.²¹

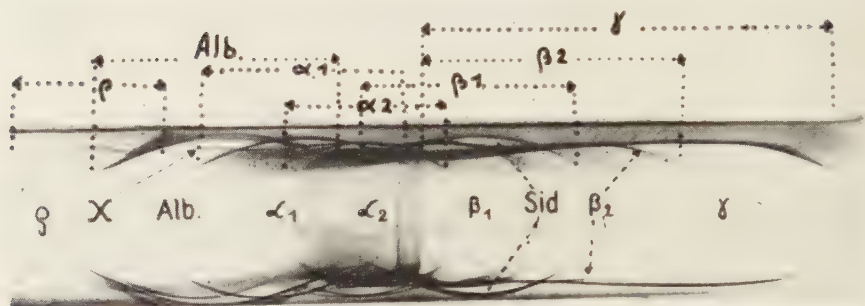


FIGURE 14. The immunoelectrophoretic analysis of normal human serum with two different horse antisera.

known. These variations can be qualitative (that is, the presence or absence of antibodies to one or another antigen) as well as quantitative (that is, different quantities of antibody directed against different antigens). In general, we use sera of hyperimmunized animals, and the best results thus far obtained have been achieved with horses and goats, which can be immunized during long periods of time and give more serum than do other animals. Moreover, horse antiprotein antibodies present the advantage of giving finer precipitation lines because of their well-known limited zone of flocculation.

Many different applications of this method can be envisaged. So far the principal applications have been the following:

- (1) Analysis of natural liquids: normal human (FIGURE 14)^{17, 48, 59} and ani-

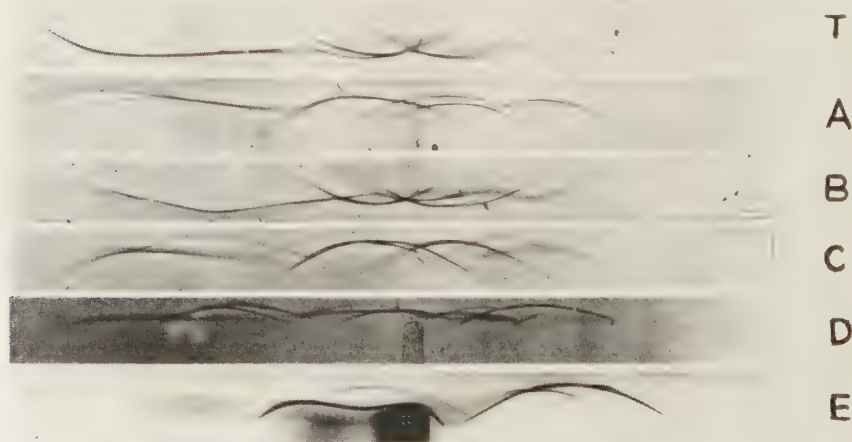


FIGURE 15. Immunoelectrophoretic analysis of human sera: T = normal, A = typical γ myeloma, B = γ myeloma with rapidly migrating peak, C = myeloma with an abnormal protein in the β zone, D = myeloma with Bence-Jones proteins, E = typical macroglobulinemia. In most patterns the arc of serum albumin is absent because of an excess of antigen.^{9, 13}

mal sera,¹⁸ cerebrospinal fluid,¹⁶ seminal fluid,³⁹ egg white,^{11, 29} pathological sera (FIGURE 15).^{9, 13, 24}

(2) Analysis of cell or tissue constituents: lens of the eye,⁵⁸ leukocytes,^{18, 52b} microbial extracts.¹⁵

(3) Embryological development: serum constituents of the human fetus,⁴⁷ and of the chick embryo.³⁰

(4) Enzymatic degradation of a protein.³³

(5) Control of purity and identification of impurities in different preparations such as: serum constituents,^{7, 17, 19, 22} and diphtheria toxin (FIGURE 16).⁴¹

(6) Studies on antibodies appearing in the serum of animals during hyperimmunization.^{18, 41, 50}

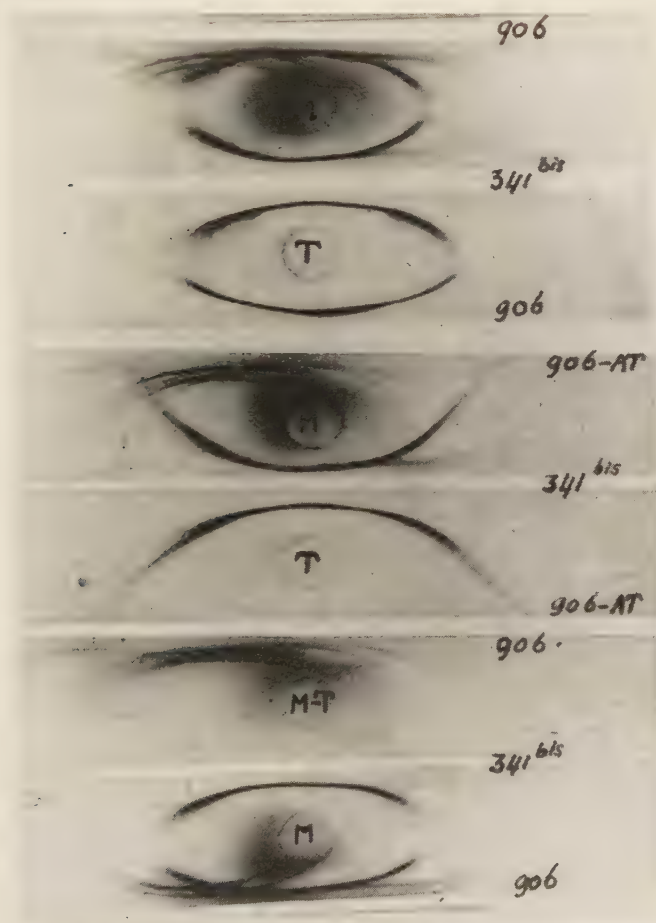


FIGURE 16. Immunoelectrophoretic analysis of diphtheria toxin preparations of different purity with horse antitoxic sera. T = pure toxin, M = initial toxin preparation, MT = initial preparation from which the toxin has been extracted, 906 = total antitoxic serum, 906-AT = antitoxic serum absorbed with the pure toxin, and 341 bis = purified antitoxin.⁴¹

Acknowledgments

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IMMUNOCHEMICAL ANALYSIS BASED ON COMPLEMENT FIXATION

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The original plan for this part of this monograph was to have experts in immunochemical methods point out the limitations in their special fields, so that others might improve their chances of escaping the pitfalls. I am reminded of the story of the child who returned home from school with a book of natural history under his arm and proudly announced to his mother that he had won it in a contest. The question was, "How many legs has an ostrich?" and the boy said that he had answered "Three." "But an ostrich has only two legs," his mother corrected. "I know," was his reply, "but all the other kids in the class guessed four!" In accepting the assignment as an expert in complement fixation, I find myself in a similar situation. There are some differences—for example, no one is certain whether the correct answer will turn out to be 2 or 1 and, while a few of us have guessed 3, and some have chosen 4, most of the class is still working on 5.

The Complement-Fixation Reaction

Complement fixation is a difficult subject to discuss in terms that have meaning for anyone but the expert, and experts hardly can be expected to listen to one another. The complexities of the subject arise from the fact that we are dealing with an indirect method. In other words, two separate and distinct reactions take place in each analysis by complement fixation. The first reaction is the combination of antigen with antibody to form an immune complex. This is the primary reaction, and the one in which we are mainly interested, since it is the quantity of antigen or antibody participating in this first reaction that we wish to measure. The second is the indicator reaction. This is an immunological reaction involving a special set of reagents. Here the antigen is the sheep red cell, and the antibody is rabbit antiserum against this cell. The immune complex formed is called a sensitized cell. This is a unique type of immune complex. It has the property of releasing into solution the hemoglobin contained in the cell if a certain group of substances collectively called complement is present. A certain quantity of complement is necessary to cause lysis of a sensitized cell, and the indicator reaction therefore serves to define the unit of complement activity.

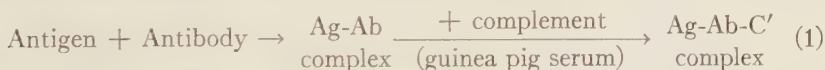
To complete the definition of the complement-fixation reaction it is necessary to state that the primary reaction inactivates or "fixes" an amount of complement that is related to the quantity of immune complex. It is then possible, by means of the indicator reaction, to measure the quantity of immune complex by determining how much complement activity remains. It should be clear

that the sensitivity of the test is determined by the quantity of complement added in the primary reaction. It is probably equally clear why discussion of this method can lead easily to utter confusion. I may add parenthetically that the points to be discussed in this paper are directed toward those of us whose intellectual endowments are insufficient to cope with complexity of this order, and who require some simplifying concepts in order to understand it.

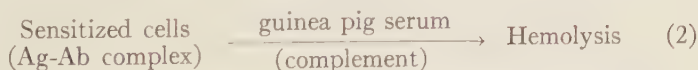
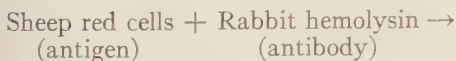
The soundest advice that one can give to those who would employ complement fixation in cancer studies is to avoid it. The mental anguish and experimental difficulties associated with this technique strongly recommend that it be avoided except in situations in which no other method is available. Unfortunately, there are several of these that come to mind immediately. For one thing, the complement-fixation method is very sensitive, and it can measure antigen or antibody at about one tenth the concentration required for the precipitation technique. For another, it can be used to study insoluble antigens such as cellular or subcellular units, or certain classes of compounds, principally lipids, that do not lend themselves readily to quantitative investigation by other methods. Finally, it is possible, although there is as yet not much evidence, that certain kinds of molecular interaction may be detected uniquely by the property of inactivating complement. If, then, we find ourselves confronted with a situation in which there is no escape from complement fixation, let us ask how we can best go about it.

Dominant Variables

Although there are many sources of information regarding the technical execution of complement fixation, the reasons for particular directives are frequently obscure. This deficiency invites each investigator to modify one or more of the many variables without sufficient appreciation of the effect this variation may have on the whole. I think we should consider such changes in separate categories. Our principal concern is with the quantities of antigen (Ag) and antibody (Ab) in the primary reaction (EQUATION 1)



If we are dealing with very small concentrations of reactants, then reaction rate also plays an important part, and this is reflected in the incubation time and temperature employed. Most of the enterprise of complement fixers is exerted in the indicator reaction (EQUATION 2)



where it is least needed. The variable constants in this reaction are the number of sheep red cells (antigen), the quantity of rabbit hemolysin (antibody), the incubation conditions, and the method of recording the observations. Some

factors, such as pH and the concentration of the important cations Ca^{++} and Mg^{++} , are sometimes left to chance. You may notice that thus far I have carefully avoided the mention of complement. The method of complement fixation was developed in 1901 by Bordet and Gengou, and it would be neither possible nor profitable to review the many variations of it that have been reported since then. What we shall present in this paper is an individual effort to extract useful, transmissible information from complement-fixation measurements. To do this we have relied heavily on what constitutes meaningful information in the study of immune reactions based on the direct method of precipitation, and we have attempted to combine this with chemical principles. This approach is empirical, and involves assumptions that might not be acceptable to those who are more theoretically inclined. For example, one unit of complement activity found in guinea pig serum is not represented by the same proportions of complement components as one residual unit of activity after fixation has taken place. If, then, the quantity of immune complex that fixes 4 of 5 units is compared with the quantity that fixes 5 of 6, these quantities will not necessarily be in the simple relation of 4 to 5. However, the proportion is close enough experimentally to provide justification for the underlying concepts. It should be stressed that this single example is an oversimplification; by means of it we wish to suggest that, in applying complement fixation, the procedure adopted need not incorporate all knowledge available from studies of immune hemolysis (the indicator reaction) *per se*.

The Comparison of Tumor Tissues and Normal Tissues

In continuing this discussion of complement-fixation analysis it will be valuable to turn to a specific example. Let us consider the problem of how to compare a tumor tissue with various normal tissues. In making this comparison, we are confronted with several methodological choices, apart from that of immunological method. These are concerned with the antiserum serving as an analytical reagent and with the preparation of antigen from various tissues. The example we have chosen is the comparison of rat lymphosarcoma with normal rat tissues. The antiserum is a first-course hyperimmune rabbit serum against the mitochondrial fraction of the Murphy-Sturm tumor.¹ The test antigens are mitochondrial fractions of the tumor and normal tissues prepared as described elsewhere.¹ We do not wish to place any special significance on the use of mitochondrial fractions. From the standpoint of complement fixation this technique is methodologically superior, since it enables us to carry out absorption studies effectively. Furthermore, this fraction contains the strongest antigens, so that the results obtained with it probably do not differ qualitatively from those observed with whole homogenate. Finally, the mitochondrial fraction can be prepared under exceptionally mild conditions, and it thus offers some increase in specificity without impairing reproducibility, a serious limitation of more extensive fractionation methods.

Complement-Fixation Method

Before presenting the results, it will perhaps be of value to describe briefly our complement-fixation procedure. We do not mean to suggest that our

method is ideal, but rather we believe that variations will best be understood against a background of specific details.

Primary reaction. To 0.05 ml. of appropriately diluted antiserum is added 0.1 ml. of guinea pig serum diluted to contain sixty 50 per cent hemolytic units per ml. (that is, between 0.07 and 0.09 ml. of undiluted guinea pig serum per ml.). This is followed by 0.1 ml. of antigen dilution in saline. The volume is brought to 0.3 ml. with saline. The reaction between antigen, antibody, and complement is allowed to proceed at 20° C. (water bath) for 2 hours.

Indicator reaction. To each tube, 0.2 ml. of a standardized 2.5 per cent suspension of sensitized sheep cells is added, and hemolysis is allowed to proceed at 37° C. for 30 minutes with mechanical shaking. The tubes are then chilled, and 1 ml. of ice-cold saline is added. After mixing, the tubes are centrifuged for 5 minutes, and the percentage of hemolysis is read in the Coleman Junior Spectrophotometer at 545 m μ . The sheep cells are standardized by adjusting a 5 per cent suspension until, after lysis with 14 volumes of distilled water, the optical density is 0.58 in a cuvette of 10 mm. light path. The adjusted suspension is then sensitized with an equal volume of rabbit hemolysin, the concentration of the latter being the lowest that will produce maximal sensitization of the cells.²

Evaluation of reactions. The degree of reaction in complement-fixation tests is judged from the extent of hemolysis, which is then frequently reconverted to a direct measure and scored as extent of fixation on a scale of 0 to +4. This method has been adopted by most workers because it is convenient and, while it can be quite useful where the experimental differences are large, it imposes severe limitations in quantitation when the differences are small. To quote from a referee's comment on one of our recent papers "... a difference ... expressed as 2 tubes ... would be regarded as barely significant." If we must consider fourfold differences as "barely significant," then very few significant observations can be made in the field of tissue antigens. What is needed is a more precise method for evaluating reactions, at least to permit consideration of differences of 1 $\frac{1}{2}$ -fold as having a reasonable degree of significance. This has been accomplished in two steps:

(1) The indicator reaction is used precisely as one uses a chemical indicator; that is, one determines the quantities of antigen or antibody that always give exactly the same hemolytic end point. For several reasons, principally those of sensitivity and the detection of interference, the end point selected is 50 per cent hemolysis (FIGURE 1). In practice, this means titrating antigen with a constant amount of antibody and complement, or titrating antibody with a constant amount of antigen and complement. With twofold serial dilutions, an end point of exactly 50 per cent hemolysis is rarely obtained. Instead, we usually find one or two tubes with partial hemolysis other than 50 per cent. We can then determine the quantity of antigen (or antiserum) for the end point by graphic interpolation from a plot of log dilution versus log unit (logit) hemolysis. In most cases these lines are straight (FIGURE 2). Such a plot offers the additional advantage of using readings of 0 or 100 per cent hemolysis, since these set limits on the slope of the interpolation line. The result obtained is thus consistent with observations made with two or three tubes, rather than

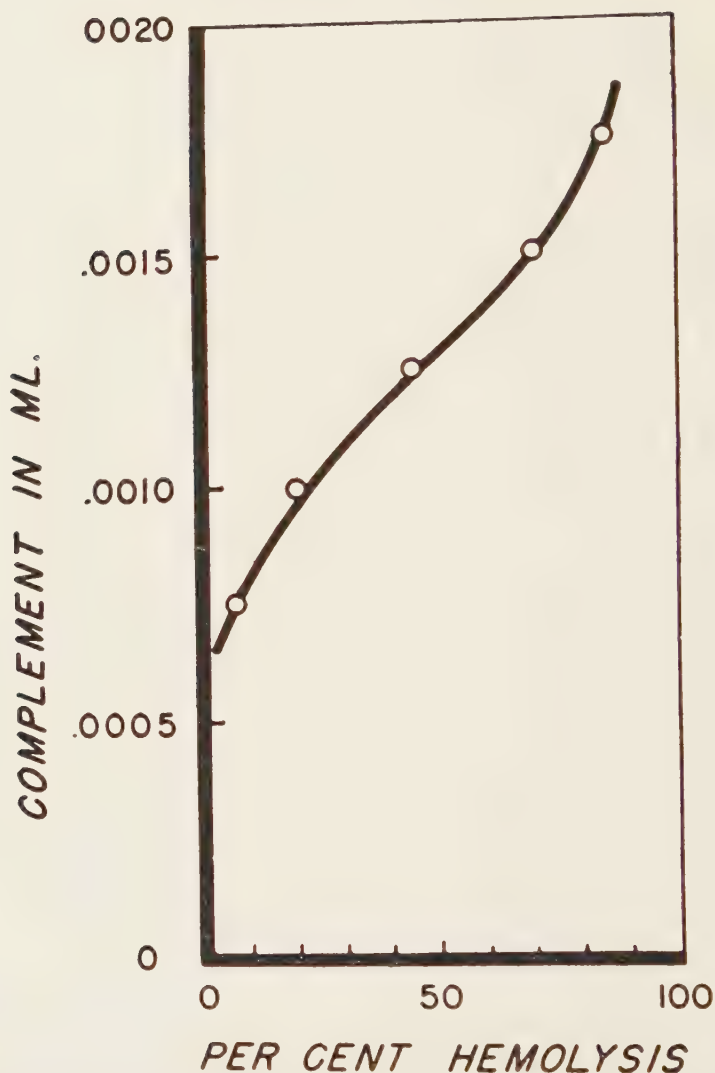


FIGURE 1. Complement titration curve showing that the region of 50 per cent hemolysis most sensitively reflects differences in quantity of complement.² Reproduced by permission of the New York State Department of Health, Albany, N. Y.

just one. Closer approximation can be made by selecting intervals smaller than twofold. Intervals greater than this are clearly not consistent with the improvement of the significance of small differences. TABLE I shows the reactivity of anti-rat lymphosarcoma serum studied in this way, the values indicating the quantities of mitochondrial fraction of tumor and nine normal tissues that react with the antiserum and a definite quantity of complement to produce an identical hemolytic end point. In this test, the quantity of comple-

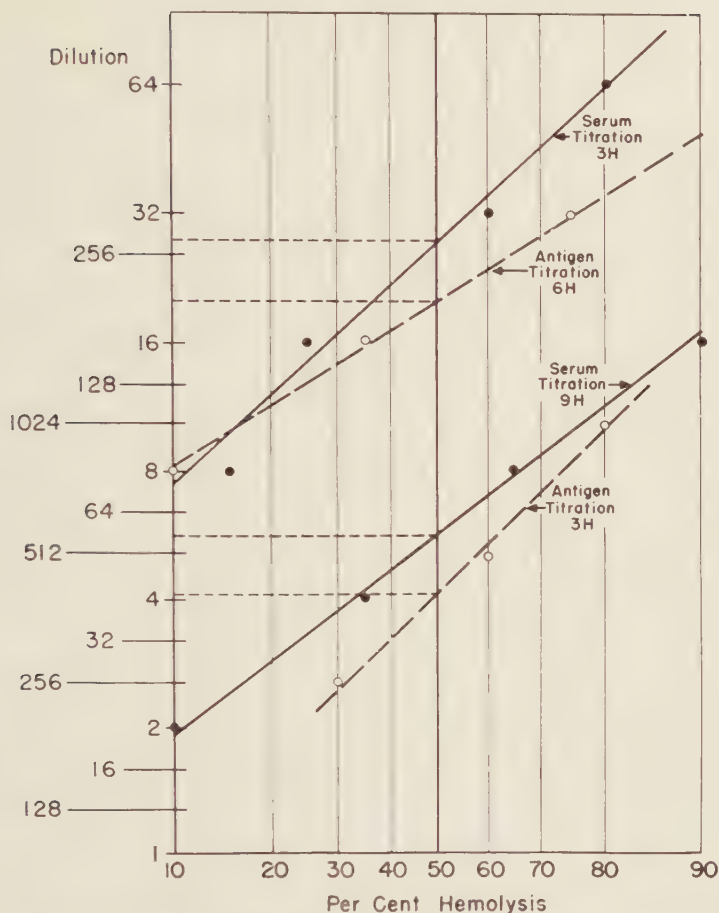


FIGURE 2. Estimation of dilution titers for 50 per cent hemolysis by graphic interpolation from a plot of log dilution versus logit hemolysis. Straight lines usually are obtained in the absence of inhibition zones.

ment determines the level of sensitivity, and I shall discuss this point in detail later. With different quantities of antiserum (Column 1 refers to a 1:5 dilution; Column 2, to a 1:10 dilution), the picture changes somewhat. Rather large changes are apparent, for example, in the relative reactivity of liver and brain tissue as compared with that of tumor. It does not require much insight to realize that finding some method of expressing the differences between tumor and normal tissues will not be easy.

(2) If we were studying soluble proteins by the precipitation technique, the suggestion immediately would be forthcoming that it is not possible to describe the relationship between cross-reacting antigens or complex mixtures in terms of the quantity of precipitate obtained with a single quantity of antigen, and

TABLE 1
REACTIVITY OF ANTI-RAT LYMPHOSARCOMA SERUM

Rat tissue—mitochondrial fraction	Micrograms of nitrogen for 50 per cent hemolysis with 6 units of complement	
	Antiserum dilution	
	1:5	1:10
Lymphosarcoma	1.36	1.58
Lung	1.03	1.12
Kidney	1.19	1.91
Spleen	1.68	1.93
Testis	1.93	2.70
Stomach	2.30	3.14
Intestine	2.48	3.04
Heart	2.44	2.66
Liver	2.53	4.75
Brain	1.63	7.15

that what is required is a complete picture of the interaction in all three zones of antibody excess, equivalence, and antigen excess. This picture of interaction would not produce any simple quantitative expression of differences, but it would reveal the conditions under which significant comparisons could best be made. Can we do the same with complement fixation?

The Block Titration: Integration into the "Isofixation" Curve

Serologists have long known that the observed reactivity is dependent on the relative proportions of antigen and antibody, and it is therefore common practice to search for optimal concentrations by means of block or checker-board titrations. In this technique a series of antigen dilutions is allowed to react with a series of antiserum dilutions; TABLE 2 shows such a block titration for the rat lymphosarcoma system. It is possible to convert the block titration into a line figure on a plot of quantity of antibody as ordinate versus

TABLE 2
BLOCK TITRATION OF ANTI-LYMPHOSARCOMA SERUM WITH HOMOLOGOUS ANTIGEN

Antigen dilution	Antiserum dilution									
	1:4	1:7	1:10	1:14	1:20	1:28	1:40	1:60	1:80	1:100
	Per cent hemolysis with 6 units of complement									
1 to 5	0	0		0		0				
1 to 10	0	0		0		0				
1 to 17			0		0		0	20	65	95
1 to 20	0	0		0		0	0	20	60	95
1 to 25			0		0					
1 to 40	0	0		0		5				
1 to 80	10	30		45		85				
1 to 160	90	100		100		100				

quantity of antigen as abscissa by adopting the method of end-point determination referred to above. You may notice that TABLE 2 is not the conventional checkerboard. It does not adhere rigidly to twofold serial dilutions, and not all squares are filled. The reason will be apparent from FIGURE 3, in which the data in TABLE 2 are integrated into the line curve. We see that the block titration of TABLE 2 is composed of four 6-tube antigen titrations at antiserum dilutions 1 to 4, 7, 14, and 28, and two 6-tube antiserum titrations at antigen dilutions 1 to 17 and 25, which are sufficient to establish fully the reactivity curve characterizing this system (FIGURE 3). Let us look at this curve more closely. In the first place, it offers the same kind of information that has been found most useful in direct methods based on precipitation, since it relates quantity of antibody to quantity of antigen *on linear scales*. In the second place, it is obtained by only a slight extension of the technique currently employed by serologists. The curve therefore represents a synthesis of well-established and actively used methods for securing and transmitting immunochemical information. The limitations compared with precipitation are still severe. Although the quantity of antigen is based on weight units, and is therefore presented in an absolute sense, the quantity of antibody is only relative. Also, whereas the degree of fixation by a given quantity of complex can be reasonably well controlled in experiments carried out simultaneously, day-to-day fluctuations are often considerable.¹⁻³ These limitations can be largely overcome by the use of adequate standards for either antigen or antibody.¹⁻³⁻⁵ The curve (FIGURE 3) may be divided into three parts: (1) a section, nearly vertical, that represents the region of antibody excess; (2) a section, nearly horizontal, that represents the region of antigen excess; and (3) the connecting curved section which represents the zone of equivalence. While classic serologic procedures have sought out the equivalence zone in order to

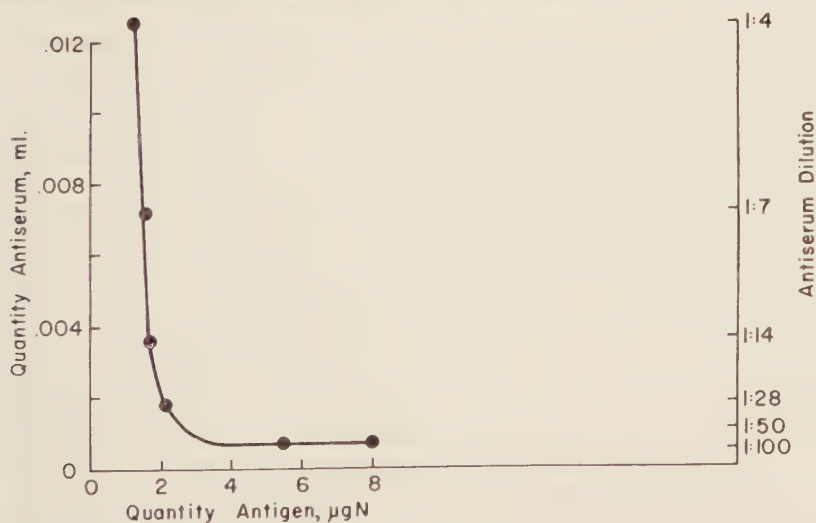


FIGURE 3. Integration of block titration of TABLE 2. Reactivity curve representing the interaction of antigen and antibody for constant hemolytic end point (50 per cent).

measure antibody with the greatest sensitivity, it will be apparent from the following discussion that this zone may not always be ideally suited for such measurements.

One question that immediately arises is why the curve does not show inhibition in the zone of antigen excess, since this is known to be one of the characteristic properties of antigen-antibody interaction. There are several reasons for this, but first I should like to discuss some other immune systems.

Isofixation Curves for Various Immune Systems

The curve just presented (FIGURE 3) describes a system in which the antigen does not satisfy any objective criteria of purity, and it would perhaps be advisable to consider the interaction of well-defined systems. Only two are available. One is the human C-reactive protein-rabbit antibody system shown in FIGURE 4.³ If we plot quantity of an absorbed rabbit antiserum versus quantity of antigen (serum containing C-reactive protein - CRP), we obtain an interaction curve that, as before, has an almost vertical section in the region of antibody excess; now, however, evidence of inhibition does appear in the zone of antigen excess. This curve takes the form of a non-rectangular hyperbola with a minimum in the zone of equivalence. The other well-defined immune system is the syphilitic antibody-cardiolipin antigen system. The curves shown in FIGURE 5 were published by Almeida

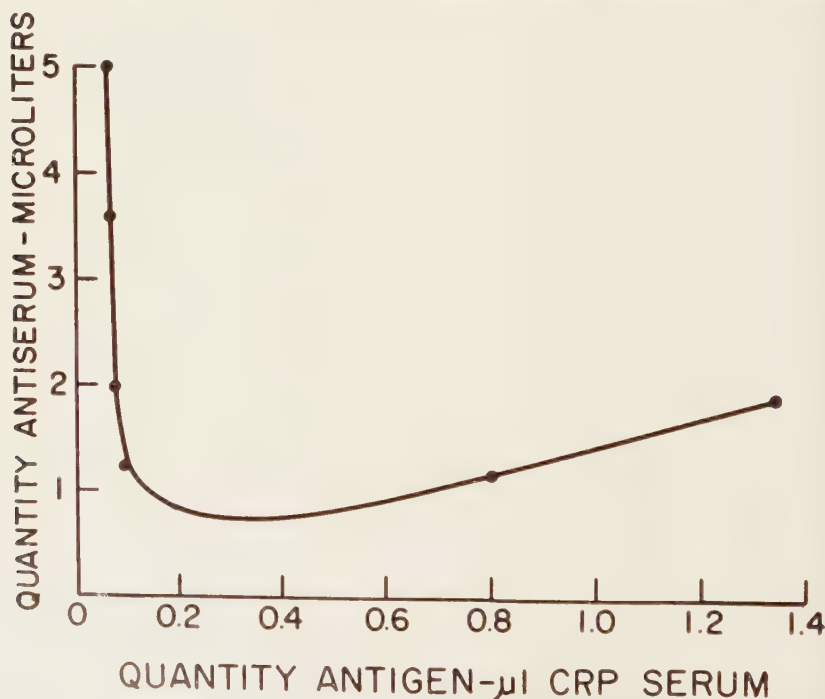


FIGURE 4. Antigen-antiserum reactivity curve for human CRP-rabbit antibody system.

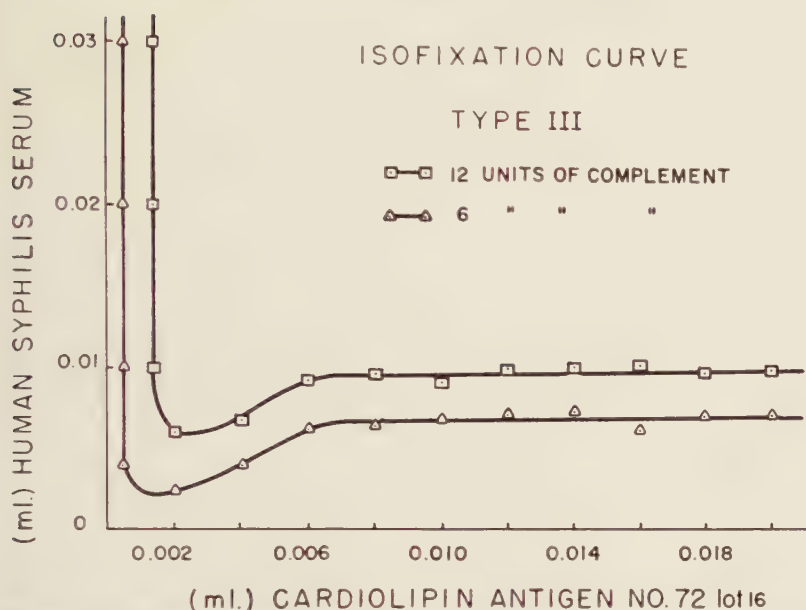


FIGURE 5. Isofixation curve for cardiolipin-lecithin cholesterol antigen-human syphilitic serum system.⁵ Reproduced by permission from *The Journal of Immunology*.

in April 1956.⁵ You will note that these correspond to the first curve we examined, with the exception of the peculiar dip in the equivalence zone. Almeida's figure shows two curves, each determined with a different quantity of complement. I shall discuss this aspect later, but while directing your attention to FIGURE 5, perhaps it will be instructive to point out one of the more intricate features of the quantitative complement-fixation test for syphilis as practiced in the United States solely by the New York State Department of Health. In this form of the Wassermann test, conditions are determined under which the quantity of antibody is related to the quantity of complement as a straight-line function. The antibody titer is then calculated as the slope of the line. The puzzling feature, almost unique for this immune system, is that a different quantity of antigen is used with each level of complement. That is, antigen is varied so that more is supplied for determinations of larger quantities of immune complex. These curves show us the reason for the simultaneous variation of two variables (complement and antigen) to determine the third (antibody). The measurement seeks out the minimum on the curve, represented as the bottom of the dip, because this point obviously conforms to the greatest reactivity of antibody for a given quantity of immune complex. For a larger quantity of complex (that is, using more complement), the minimum is displaced to a position of increased antigen. These relationships were determined by the Maltaners¹³ from empirical considerations alone. Almeida's curves also show us that if a larger quantity of antigen is used, the linear relationship between antiserum and complement can be obtained without resorting to variations in antigen, but only at the cost of decreasing sensitivity.

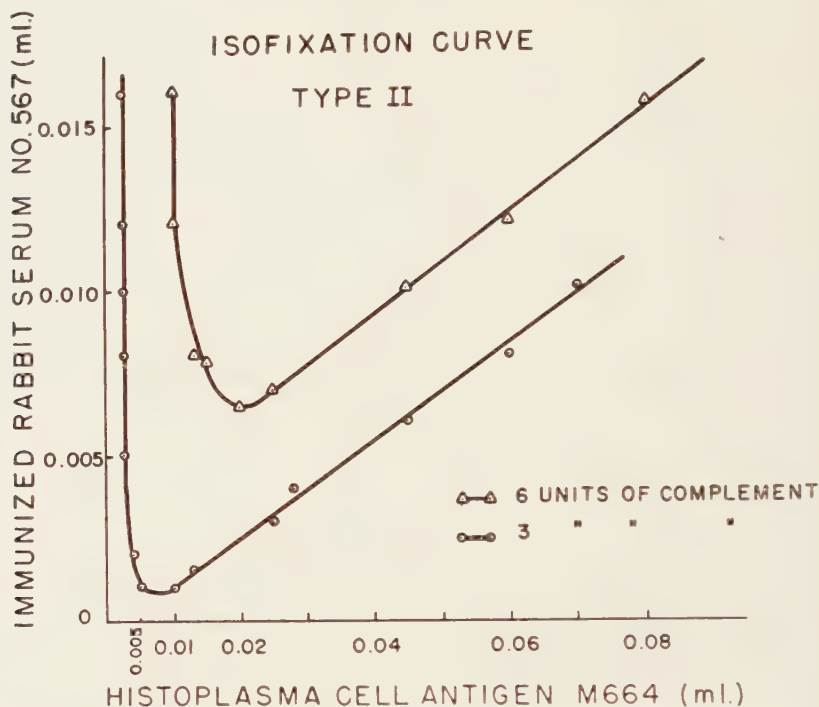


FIGURE 6. Isofixation curve for histoplasma cell antigen-rabbit antibody system.⁵ Reproduced by permission from *The Journal of Immunology*.

In other words, the determination can be made in the region of antigen excess where the curves are parallel to the antigen axis, but in this region measurements of antibody are only half as sensitive. It is probable that most other modifications of the Wassermann reaction operate in this zone. Our other examples are cases of practical rather than well-defined antigens, but they are equally instructive. FIGURE 6 shows Almeida's study of the *Histoplasma* cell antigen-rabbit antibody system, for which there is exceptionally marked inhibition by excess antigen. This type of curve, which is similar to that of the human CRP-rabbit antibody system is also observed, according to Almeida,⁶ with various fungal, viral, and human serum protein antigens and their corresponding human or rabbit antibody. Finally, in FIGURE 7 we return to the simplest and most common type of curve. In addition to lymphosarcoma and many other tumor tissue antigen-rabbit antibody systems,^{1,7} it is also characteristic for tubercle and leprosy bacillus antigens and human antibody, Cocksackie virus antigen and mouse antibody, poliomyelitis virus antigen and horse antibody, blastomycosis and pertussis antigens and the respective rabbit antibody.⁵

These curves therefore are generally applicable for the characterization of antigen-antibody interaction. They supply information that permits recognition of the zonal phenomena of immune reactions, even when associated with

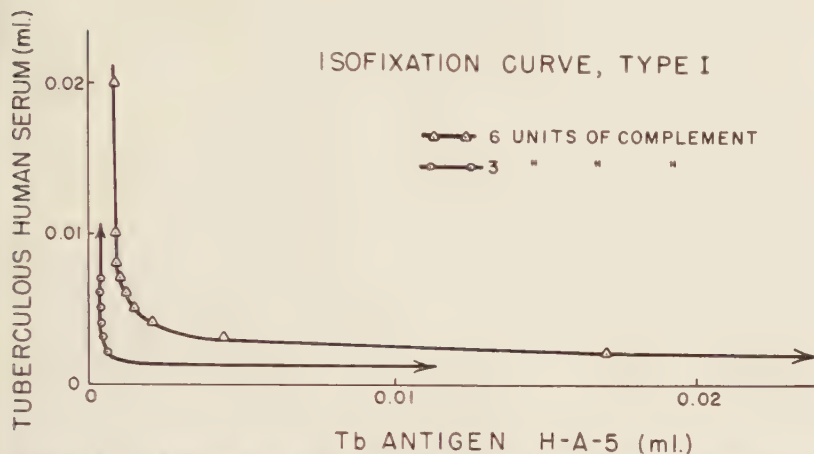


FIGURE 7. Isofixation curve for tubercle bacillus antigen-human antibody system.⁵ Reproduced by permission from *The Journal of Immunology*.

antigenic complexity, anticomplementary interference, and cross reactions. The earliest study of immune interactions in this way was employed by Maltaner and his co-workers to show that zones of inhibition were not present in either antibody excess or antigen excess for the system tubercle bacillus antigen-horse antibody. Almeida has used the method for many years in order to determine optimal antigen concentrations in standardizing quantitative complement-fixation tests for antibody (that is, in seeking out the zone of equivalence).⁵ Our own studies, which are concerned principally with antigenic comparisons rather than antibody measurements, brought us to an appreciation of the capacity of this method of representation to reveal and to transmit the kind of information that has proved so useful in precipitation.¹

Isofixation Curves Derived from Precipitation Curves

It is illuminating to consider the relationship of these curves, based on complement fixation, to those based on precipitation. For this purpose, we have selected a model system proposed by Pauling *et al.*⁹ This model was chosen, not because it offers any theoretical advantage over those proposed by others, but rather because with this model there was presented a family of curves relating quantity of precipitate (rather than antibody) to quantity of antigen, each curve representing a different quantity of antibody (FIGURE 8). The equation for this family of curves is

$$AB_{(pp)} = A_{total} - 1 - \frac{2}{3} \{A_{total} - B_{total} + [3 + (A_{total} - B_{total})^2]^{\frac{1}{2}}\} \quad (3)$$

where

$AB_{(pp)}$ = quantity of precipitate

A_{total} = quantity of antigen added

and

B_{total} = quantity of antibody added.

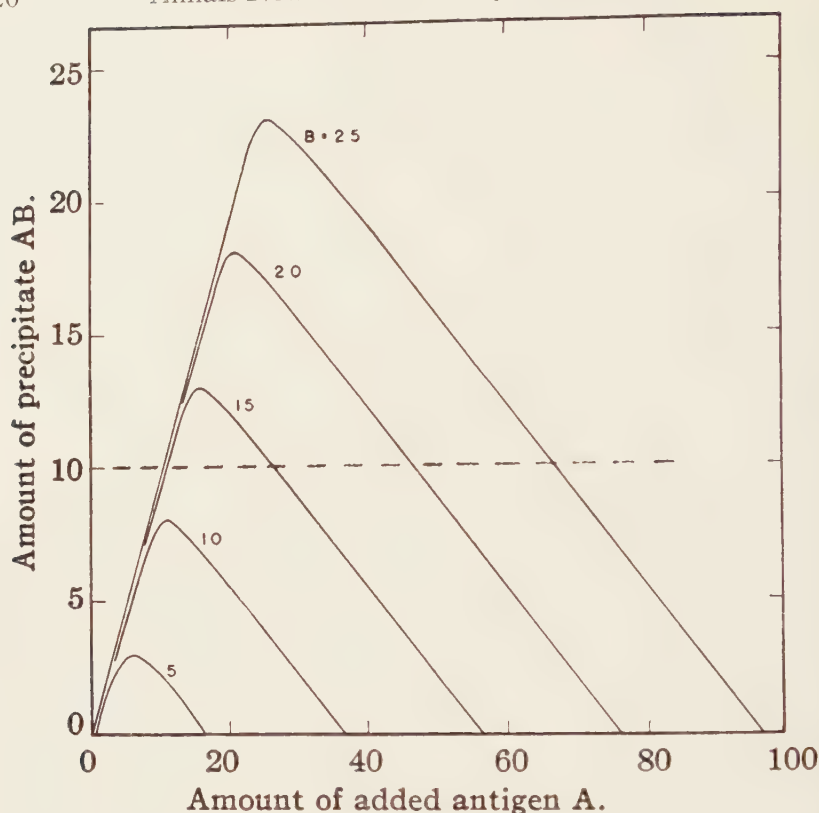


FIGURE 8. Theoretical curves showing amount of precipitate AB as function of the amount of antigen A for antisera with varying antibody concentration $B = 5$ to 25 . The values of the constants used are $S = 1$ and $K = \frac{1}{2}$.⁹ Reproduced by permission from *The Journal of the American Chemical Society*.

To adopt this model we must first redefine some of the terms. The quantity of precipitate may be interpreted as "immune complex that fixes complement," whereas "soluble complex" is equivalent to "immune complex that does not fix complement." The mechanics of complement fixation, based on adding a certain quantity of complement and proceeding to the same hemolytic end point, are equivalent to considering precipitation technique in the sense of determining how much antibody interacts with how much antigen to produce an identical quantity of precipitate.

If, in EQUATION 3, we let $AB_{(pp)}$ be constant, the equation reduces to

$$y = \frac{(x + a)^2 - 4a^2 + 4}{4(x - a)} \quad (4)$$

where

$$y = B_{total}$$

$$x = A_{total}$$

and

$$a = AB_{(pp)} + 1$$

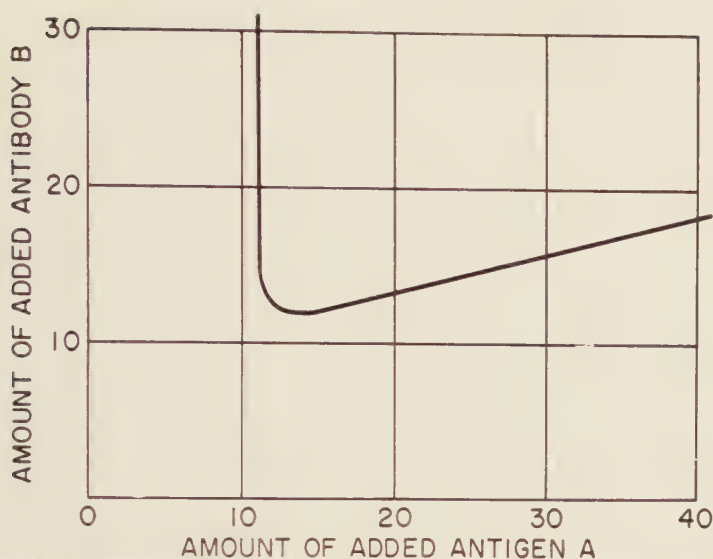


FIGURE 9. Theoretical isofixation curve showing amount of antibody B that interacts with the amount of antigen A to produce a constant quantity of immune complex AB. The values of the constants used are $S = 1$, $K = \frac{1}{2}$, and $AB = 10$ (dotted line of FIGURE 8).

EQUATION 4 describes a hyperbola with asymptotes $x = a$ as $y \rightarrow \infty$ and $y = x + \frac{2a}{4}$ as $x \rightarrow \infty$; there is a minimum at $x = a + 2$, $y = a + 1$ (FIGURE 9).

The similarity of this curve to those obtained in actual practice with some systems (FIGURES 4 and 6) is seen readily. The family of curves shown in FIGURE 8 describes the behavior of a hypothetical model in which excess antigen markedly inhibits the formation of specific precipitate. The complement-fixation curve for this system (FIGURE 9) shows a much less marked effect in the zone of antigen excess. Therefore this difference is not to be explained by a difference in the capacity of excess antigen to inhibit immune complex formation, but is inherent in the method of measurement. The theoretical curve shown in FIGURE 9 describes the zone of antibody excess by an almost vertical line, which reflects the fact that for the model the curves (FIGURE 8) are almost superimposable in this zone. Departures from this behavior for actual systems may provide criteria by which one can judge heterogeneity of antibodies, antigenic complexity, anticomplementary interference, or insufficiency of antibody for "antibody excess." Similar interpretations are possible, but theoretically less secure, from comparisons in the zone of antigen excess. In any case, it is possible to see from the model that isofixation curves, considered by Almeida⁵ to be of three different types, are really variations of a basic curve that is consistent with properties of immune complex formation determined by the direct method of precipitation. The variations are analogous to those encountered in precipitation curves. We all know the distinctive features of different immune systems revealed by such curves, where a broad plateau may occur either as an inherent property of the interaction or from

the presence of multiple immune systems. To make the analogy mathematically complete, the maximum for the *immune complex formation curve*, and the minimum for the *complement fixation curve* occur at the same concentration of antigen.

We may conclude our description of the usefulness of isofixation curves for studying complement fixation by referring to FIGURE 10. The two curves indicate the interaction of two different antisera with the same antigen. Let us recall that complement-fixation tests that employ a single quantity of antigen (for titration of different antisera) or a single quantity of antiserum (for titration of different antigens) give us only one point on the curve. Then, if we arbitrarily were to select the quantity of antigen shown at x , antiserum A might appear to give a specific reaction with respect to antiserum B. If the antisera were compared by carrying out antigen titrations (a not infrequent practice), then the employment of a dilution of antiserum corresponding to a would indicate that antiserum A was more reactive than B; at dilution b , both antisera would appear equally reactive; whereas at dilution c , antiserum B would appear to give a specific reaction with respect to antiserum A. Although the inherent risk associated with interpreting comparisons based

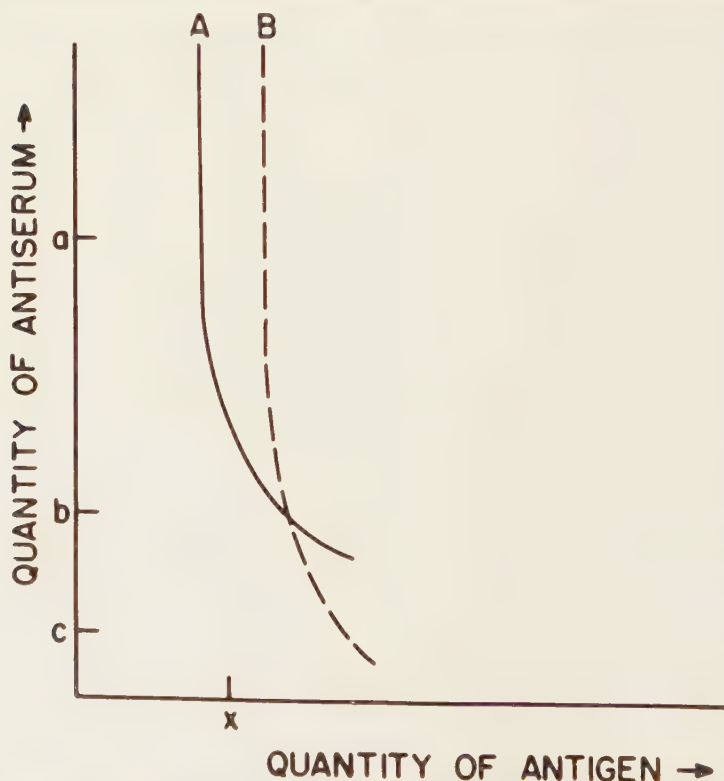


FIGURE 10. Isofixation curves so related that errors may arise if a single quantity of antigen or a single dilution of antiserum is used for evaluation. See text for explanation.

on one point is evident and has been pointed out previously,¹⁰ the substantial experimental effort involved in complement-fixation analysis has prevented any widespread acceptance of the principle.

Antigenic Differences Revealed by Partial Absorption

Let us return to the problem of comparing lymphosarcoma with normal tissues. In FIGURE 11 we see the isofixation curves of rabbit anti-rat lymphosarcoma serum with the mitochondrial (M) fractions of the homologous rat lymphosarcoma and seven normal tissues of the rat: lung, testis, spleen, intestine, kidney, liver, and brain. The differences are small with larger quantities of antiserum, as noted in TABLE 1. It is also apparent that to find a simple method of describing the observed differences between tumor tissues and normal tissues is hardly possible. One must reformulate the problem, which in turn requires redefining the objectives. Instead of seeking to answer the question, "Are specific antigens present in tumor tissue?" and considering every indication of cross reaction as evidence of nonspecificity, we are forced to ask the more limited question, "How do we prepare a reagent to distinguish between two different tissues, and how shall we use it to maximum advantage?" It is evident that by selecting the proper dilution of the antiserum at hand, a specific reagent is available for six tissues compared with brain or liver. Within the group comprising lymphosarcoma, lung, testis, spleen, kidney, and intestine, the problem is much more difficult. The solution, if there is one, lies in suitable absorption of the antiserum to remove the undesirable reactivity. You will note from FIGURE 11 that lung tissue is most closely related antigeni-

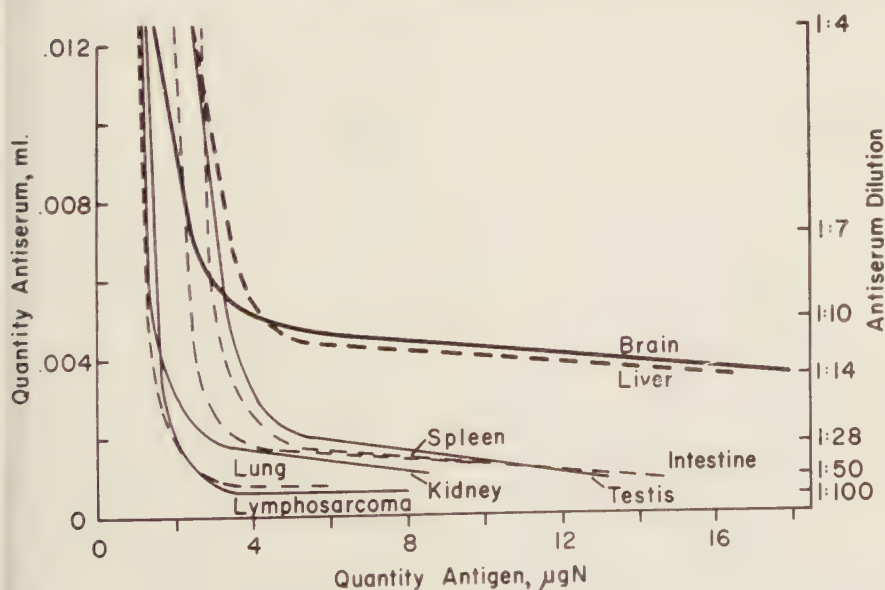


FIGURE 11. Isofixation curves for rat tissue M fractions with rabbit antiserum to rat lymphosarcoma.

cally to lymphosarcoma; indeed, the two are almost indistinguishable with untreated antiserum. Reference to TABLE 1 shows that, in point of fact, the antiserum reacts even more avidly with lung tissue than with lymphosarcoma in antibody excess. It is not unreasonable to assume that if a reagent can be made to distinguish tumor tissue from lung tissue, then it will be possible to prepare one that reveals even larger differences between tumor and the other normal tissues.

Absorption has not found its proper place in complement-fixation studies, principally because absorbed antisera are anticomplementary (AC). This residual AC activity, if not removed, seriously restricts interpretation of complement-fixation tests. The AC activity is almost certainly attributable to soluble, complement-fixing immune complex and, with the newer laboratory tools, this source of interference can be eliminated completely. With lipid antigens and washed M-fraction antigens, this can usually be accomplished by centrifuging at $20,000 \times g$ for 1 hour. With soluble antigens, $100,000 \times g$ for 2 hours usually suffices.³

The results of graded absorption of the antiserum with increasing quantities of rat lung M fraction are presented in FIGURE 12. In the panel at the left, which shows residual reactivity with the absorbing antigen, it can be seen that as we increase the quantity used for absorption, we observe the expected decrease in reactivity of the treated antiserum. Not only is the *titer* of the serum decreased but, since the more reactive antibody is removed first, the *avidity* of the remaining antibody also is diminished greatly. This is shown by the progressive increase in the "minimum detectable antigen" as antibody is removed. These changes in reactivity occur to a much lesser degree with

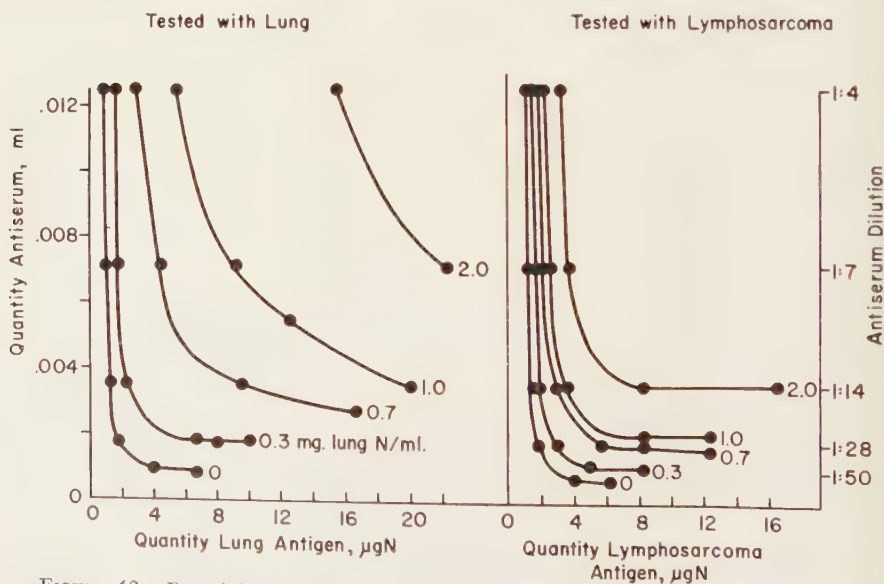


FIGURE 12. Reactivity of anti lymphosarcoma serum after partial absorption with rat lung. The numbers on the curves indicate the quantity of antigen used for absorption in mg. N lung M fraction per ml. antiserum.

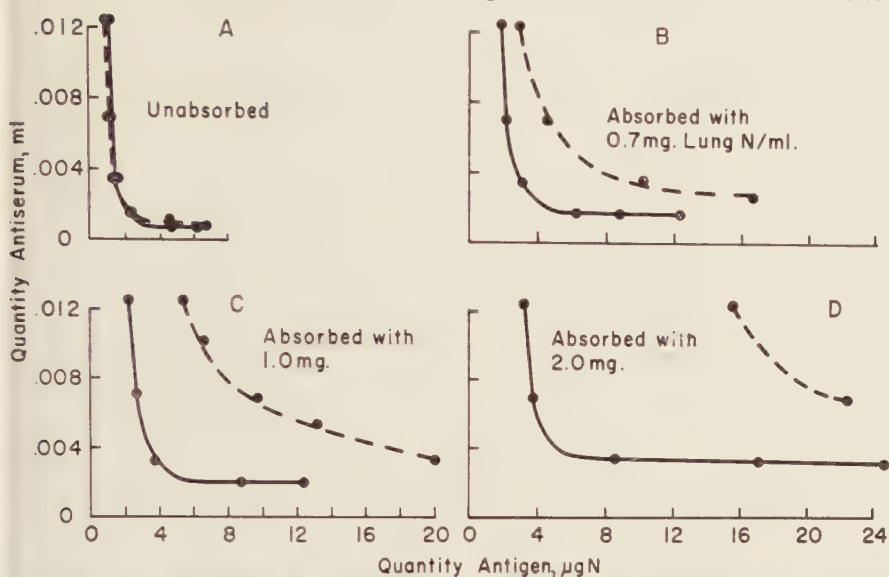


FIGURE 13. Reactivity of anti-lymphosarcoma serum after partial absorption with rat lung. The solid line shows reactivity against lymphosarcoma, and the broken line shows reactivity against lung.

respect to the homologous lymphosarcoma antigens (FIGURE 12, right panel) and the effect is even more clearly brought out in FIGURE 13, in which the absorbed antisera are individually compared with respect to their reactions to the two antigens. With partially absorbed sera, the differences become more marked until an antiserum is obtained that, when properly diluted, will react six to eight times more selectively with lymphosarcoma than with lung tissue. In the process we were forced to sacrifice a considerable part of the original reactivity of the antiserum, since the minimum quantity of detectable sarcoma antigen is three to four times greater with the most specific reagent than with untreated antiserum (compare graphs A and D in FIGURE 13). If indiscriminate amounts of lung antigen were used for absorption, the sensitivity of the resulting reagent might be depressed still further, possibly until the very differences we seek would no longer be detected. The method of graded absorption of antisera, combined with the use of isofixation curves, permits a systematic exploration of the conditions necessary for converting a cross-reacting antiserum into a specific chemical reagent. It is clear that the examination of differences between normal and tumor tissues requires a more discriminating tool than the conventional type of complement-fixation study, and the source of many limitations inherent in older methods may be discerned readily.

Sensitivity Versus Nonspecific Interference

The discussion to this point has been concerned primarily with interpretation and transmission of complement-fixation data. We have now arrived at the very heart of the matter. Given a method whose sensitivity may

be varied at will, but which is subject to multiple, ill-defined sources of interference, what degree of sensitivity is justifiable in a particular experimental situation? There is no categorical answer to this question, which touches as much on the purpose of measurement as it does on sources of error. We shall discuss several methods of varying the sensitivity of the reaction, and how best to determine the presence of interferences.

Let us recall Almeida's studies in which isofixation curves were determined with two different quantities of complement. Each curve showed different values for "minimum quantity of antigen" and "minimum quantity of anti-serum." The values are smaller, and thus sensitivity is higher, with less complement. This is illustrated more extensively in FIGURE 14, which shows the curves for rat lymphosarcoma M-fraction rabbit antibody determined

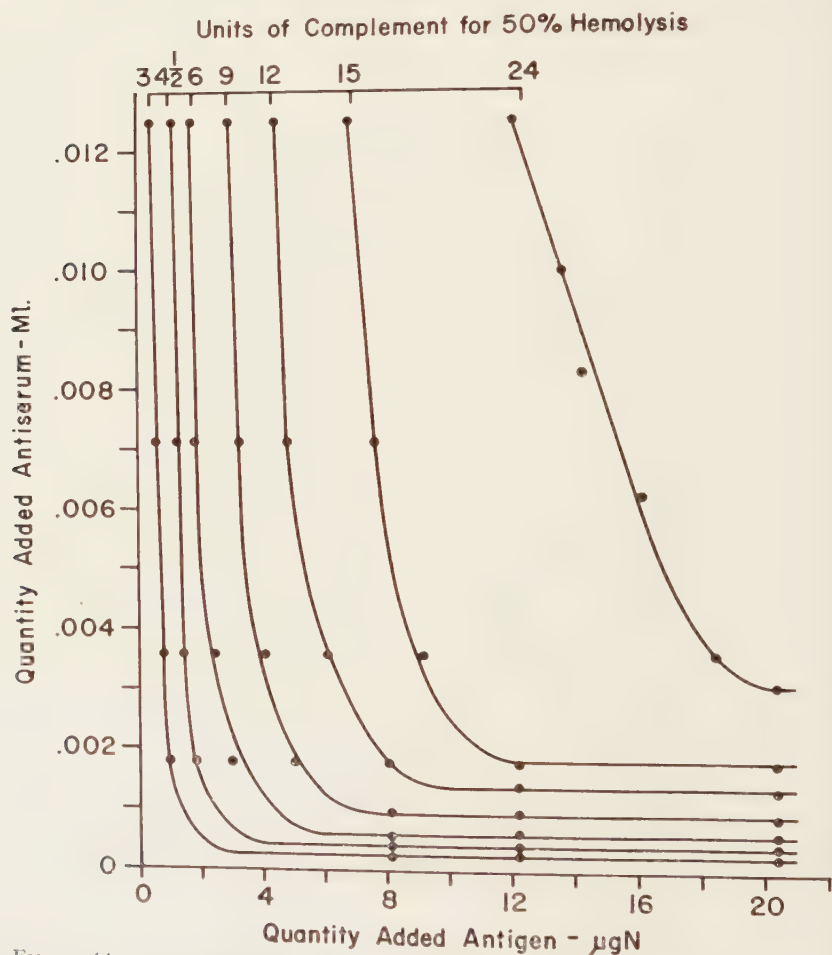


FIGURE 14. Isofixation curves for rat lymphosarcoma M fraction-rabbit antibody system obtained with different amounts of complement. The unit of complement is equal to 0.00125 ml. of guinea pig serum.

with seven different quantities of complement (3, 4½, 6, 9, 12, 15, and 24 units, the unit being equal to 0.00125 ml. of guinea pig serum). In practice, complement-fixation tests are usually carried out with a single quantity of complement, defined by the indicator reaction. The quantity of complement, expressed in such terms as "two 100 per cent units" or "four 50 per cent units" is in turn variable from one laboratory to the next, depending on the conditions adopted for measuring immune hemolysis, in particular, the number of red cells and the use of the important cations, Ca^{++} and Mg^{++} .¹¹ We have, therefore, four methods of increasing sensitivity: (1) reducing the number of red cells, (2) adding Ca^{++} and Mg^{++} , (3) raising the per cent hemolysis taken as end point, or (4) decreasing the excess quantity of guinea pig serum added in the primary reaction. These methods are not independent, since they achieve their purpose by a common mechanism, namely, reducing the quantity of complement to be inactivated by immune complex in the primary reaction. It would thus be entirely feasible (and highly desirable) to standardize the indicator reaction so that approximately the same quantity of guinea pig serum would universally serve as a unit. Under the present chaotic scheme, information on the "number of units" employed in a test gives us no more than an idea of whether nonspecific interference will be picked up by the controls; it tells us nothing about the quantity of immune complex formed. The dissociation of fixation in the primary reaction from immune hemolysis in the indicator reaction can be appreciated more readily from the following example.

C-reactive protein in a serum specimen was titrated with excess antibody in the presence of 0.00516 ml. of guinea pig serum. The indicator reaction required 0.00090 ml. of guinea pig serum for 50 per cent hemolysis in the presence of Ca^{++} - Mg^{++} -veronal buffer¹² and 0.00129 ml. without added cations. The test was therefore run with 5.75 units in one case and 4.00 units in the other. The titers (reciprocal of antigen dilution) of the CRP specimen were 1150 and 920, respectively. The greater sensitivity (that is, the smaller quantity of measured complex) in the presence of a larger number of "units" shows that the hemolytic unit per se is no indicator of the quantity of immune complex. To digress for a moment, this example needs a further correction, since the end point of 50 per cent hemolysis in the titration does not represent inactivation of exactly the same quantity of guinea pig serum in the two cases. In the presence of Ca^{++} - Mg^{++} -veronal buffer, the immune complex must remove complement activity corresponding to $(5.75 - 1.00) \times .00090 = .0043$ ml. of guinea pig serum. In the second case, the quantity is $(4.00 - 1.00) \times .00129 = .0039$ ml. In the presence of Ca^{++} and Mg^{++} , 20 per cent less antigen fixes 10 per cent more complement, and the cations therefore not only activate hemolysis, but also fixation in the primary reaction. The diabolical nature of indirect methods in general, and complement fixation in particular, is disclosed by the realization that the same effect would be produced by introducing some nonspecific interference.

Interference Not Detectable in Controls

The choice of a level of sensitivity for any analytical procedure is determined by the requirements of the scientific problem, availability of reagents, con-

venience of operation, and the nature of nonspecific reactions and other interferences. In complement fixation, the most important consideration must be given to nonspecific reactions. When these are detectable from the effect of antigen or antiserum alone on complement, they are grouped under the term "anticomplementary activity." They may *not* be detectable in this way, and then it is far more difficult to discriminate between true immune complex formation and nonspecific fixation, just as it is sometimes difficult to determine whether a given precipitate is "specific."

It is interference of this sort that presents the major obstacle to obtaining analytical results by complement fixation. Two separate methods have been proposed for surmounting this difficulty. Heidelberger and his co-workers¹² have recommended the use of a large quantity of complement in the test. The basis for this is simple and obvious: namely, by carrying out the reaction at a higher level, the effect of nonspecific reactions is avoided or kept at a minimum. In other words, specificity is made more secure by reducing sensitivity. Economy of effort is retained since the tests involve a single quantity of complement. The loss of sensitivity is, however, too severe for most studies involving tissue antigens. In most cases, a compromise is possible and, from our experience, tests based on the removal of complement activity corresponding to 0.0045 to 0.0075 ml. of guinea pig serum do not suffer much from nonspecific interference.

An independent method of dealing with the problem without sacrificing sensitivity was developed by Maltaner and his co-workers much earlier.^{6, 8, 13, 14}

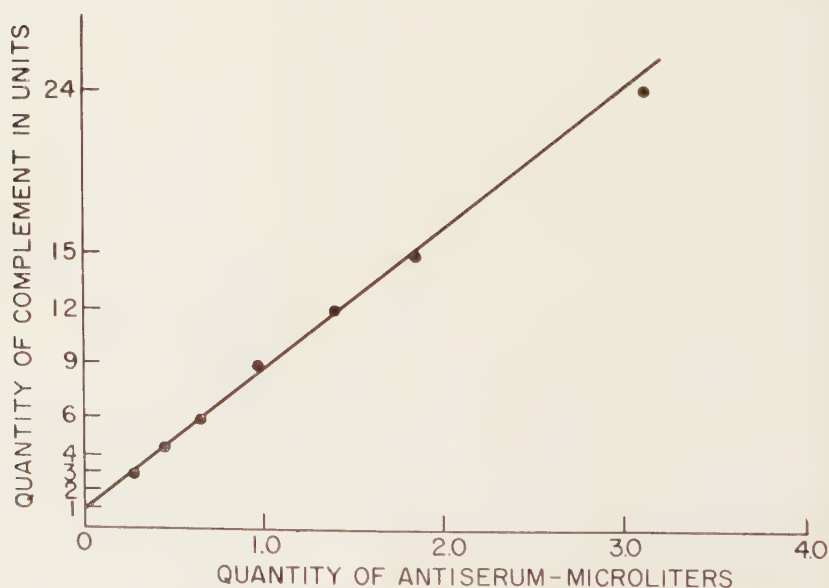


FIGURE 15. Quantity of complement plotted against quantity of antiserum, showing the linear relationship. The data are taken from the iso-fixation curves of FIGURE 14 at 20.4 μ g. of antigen N. Note the intercept of 1.0, which indicates the absence of nonspecific interference.

Their technique was to determine conditions that would give linear relationships between quantity of antiserum and complement, or quantity of antigen and complement. The antibody titer (their studies dealt principally with determination of antibody) was determined by the slope of the line, which served to eliminate reactions whose capacity to inactivate complement was less than that of immune complex. They established these relationships empirically; Almeida⁵ recently has shown how they may be selected immediately from isofixation curves. Let us look more closely at this method with the data presented in FIGURE 14. FIGURE 15 is a plot of quantity of complement versus quantity of antibody determined with 20.4 μ g. of antigen N. Antiserum is proportional to complement up to 15 units. A significant deviation appears at 24 units, indicating a deficiency in the fixing capacity of immune complex for this quantity of complement. The intercept of this line on the complement axis is 1, from which we may conclude that there are no interfering reactions. FIGURE 16 shows a plot of quantity of complement versus quantity of antigen determined with 0.0125 ml. of antiserum. Antigen is proportional to complement up to 12 units. Significant deviation occurs at 15 units, and is much larger at 24 units. From the interaction curve for 24 units (FIGURE 14) we can predict this result because of the deviation of the slope in antibody excess from that of the other curves. The line intercepts the complement axis at 2.3 units, indicating some interference with comple-

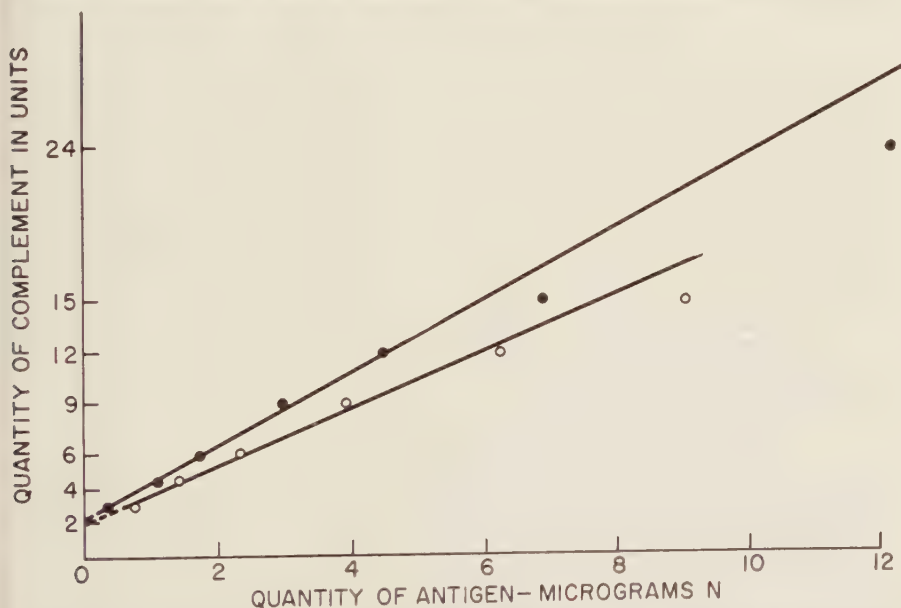


FIGURE 16. Quantity of complement plotted against quantity of antigen, showing the linear relationship. The data are taken from the isofixation curves of FIGURE 14.

Symbols: the closed circles show the data obtained with 0.0125 ml. of antiserum (1:4 dilution); the open circles show the data obtained with 0.0036 ml. of antiserum (1:14 dilution).

By extrapolation, the intercepts are found to be 2.3 and 2.0, respectively, which indicates a low degree of nonspecific interference.

ment attributable to antigen. The lower line (FIGURE 16), determined with 0.00036 ml. of antiserum, also shows proportionality up to 12 units of complement, but sensitivity is decreased because of the smaller quantity of antibody. The difference in sensitivity reflects the deviation of the curves (FIGURE 14) in antibody excess from the vertical. This method, although best for practical application to specific problems, is laborious, since it requires determinations with at least 2 different quantities of complement, and is not altogether suitable for problems in which the nature of the antigens and the level of antibody are both poorly defined (compare FIGURE 11).

Interference Detectable in Controls

The interference resulting from detectable anticomplementary effects of antigen or antibody is also controlled by the above methods. As sensitivity is increased, it becomes increasingly important to determine such interference, despite our inability to estimate its degree quantitatively. In this respect complement fixation is a much more complicated instrument than indirect *chemical* methods, because the indicator reaction is limited to a narrow range of complement activity. The presence of only 1.5 hemolytic units of activity can increase hemolysis from 50 to 95 per cent. Therefore, the loss of 0.5 units from a 2-unit test, of 2.5 units from a 4-unit test, or of 4.5 units from a 6-unit test, as a result of anticomplementary activity of the reagents, will go undetected. It is essential that controls for AC property be designed to acknowledge this fact, and that the quantity of complement used in the control be much less than that used in the test. The important advantage of using Ca^{++} - Mg^{++} activated complement is clearly apparent here, since it permits a smaller quantity of complement to serve as a unit, and therefore permits a large differential between the controls and the test. We are not able to estimate the quantitative influence of AC property on the test itself since, in the competition for complement, the relative rates of fixation by immune complex and "AC property" are not known. Our security must lie, therefore, in performing the reaction at a level as far removed from that of known interference as is consistent with the requirement for sensitivity. It is not in the interest of prudent investigation to conceal the difference as, for example, by determining the unit of complement activity (indicator reaction) in the presence of anticomplementary antigen.¹⁵

Epilogue

The discussion of complement-fixation analysis presented in this paper is incomplete in several aspects, among which may be mentioned the effects of incubation time, the stability of reagents, the relationship between observed hemolysis and the ratio of immune complex to complement, and, more generally, the studies of many other investigators. We have preferred to emphasize the more severe impediments in applying complement fixation to analytical problems and to suggest methods for their circumvention. It is perhaps not without value to add a philosophical note. We have progressed beyond that stage of biological science where it is sufficient to know that a

difference exists between two things. We must assume the responsibility for exploiting the difference in some way, which requires first, that we know the magnitude of the difference and, second, that we direct our efforts to discovering what lies at its root. This is especially true in studying antigenic relationships among the cellular constituents of normal and abnormal tissues. Interpretations of results obtained at an early experimental stage with trace amounts of antibody, grossly impure antigens, and sources of substantial interference are unreliable. They are equally unreliable for the investigator who attributes significance to *any* differential and for the authority who equates cross-reactions with nonspecificity. The solution can lie only in deeper penetration into the problem.

Acknowledgment

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It is a pleasure to acknowledge the skillful technical assistance of Mary Mejac, Evelyn Abeshouse, and Bernard Schiffer.

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VIRUS-NEUTRALIZATION TESTS: IMPLICATIONS AND INTERPRETATIONS

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When a mixture of virus and serum is inoculated in a susceptible host, only one question is asked: "Will the virus infect?" If the host shows the virus to be infective, it is assumed that the serum did not neutralize it. If the host shows the virus not to be infective, it is assumed that the serum did neutralize it. A further assumption is then made; namely, that the serum contains antibody against the virus.

In these terms, one question and one answer, the virus-neutralization test appears straightforward and unequivocal. As we shall see, this is not the case, and the complex procedure provides traps for the unwary. Valuable and at times essential though the neutralization test is, it makes demands on skill and knowledge if fallacious interpretations are to be avoided.

With one virus-serum mixture and one susceptible host, a decision as to infection depends on some end point. The end point often has an all-or-none aspect as, for example, death or survival, or paralysis or none. Death may indicate that the virus infected, but it does not show that all of it was infective. Similarly, survival indicates that some of the virus was inactive, but it does not show that none of it was infective. Thus, a test with an all-or-none end point depends on a threshold response with little quantitative meaning.

Numerous improvements are easily made. First, a number of host individuals can be given the virus-serum mixture. This adds to certainty, as when all die or survive, but it does not tell more as to the amount of virus inactivated. Second, one variable, either the amount of virus or serum, can be introduced and a series of mixtures tested, each in a group of hosts. If the amounts of both the virus and serum are known, a semiquantitative result emerges. Third, a percentile rather than an all-or-none end point sometimes can be used, and the extent rather than the fact of the infection estimated in each group.¹ The question asked now of the test has one of two forms: (1) how much virus can the serum neutralize, or (2) how little serum can neutralize the virus?

The test can be improved still further by using two variables simultaneously; that is, different amounts of virus and different amounts of serum (TABLE 1). This is the "chessboard" procedure which requires a number of series of mixtures. When each is tested in a group of hosts and a percentile end point is used, some additional questions may be asked.¹ These are: (1) what is the largest quantity of virus the undiluted serum can neutralize, (2) what is the smallest quantity of serum that can neutralize a given amount of virus, and (3) what is the relation between the quantities of virus and serum at the end point? The answer to this last question is not simple, and it bears heavily on the interpretation of neutralization tests.¹⁻⁷

When the logarithm of the serum dilution is plotted against the logarithm of the amount of virus neutralized, a linear relation becomes evident (FIGURE 1).

TABLE 1

RESULTS OF NEUTRALIZATION TESTS WITH DECREASING QUANTITIES OF THE PR8 STRAIN OF INFLUENZA A VIRUS AND CONVALESCENT HUMAN SERUM²

Virus dilution	No serum	Serum dilution							Virus neutralized
		1:5	1:25	1:125	1:625	1:3,125	1:15,625	1:78,125	
10 ⁻¹	—	5S 1D	6D	6D	6D	—	—	—	10 ^{5.6}
10 ⁻²	—	6S	6S	6D	6D	6D	—	—	10 ^{4.6}
10 ⁻³	—	—	6S	6S	6D	6D	6D	—	10 ^{3.6}
10 ⁻⁴	6D	—	—	6S	6S	6D	6D	6D	10 ^{2.6}
10 ⁻⁵	6D	—	—	—	6S	6S	6D	6D	10 ^{1.6}
10 ⁻⁶	6D	—	—	—	—	—	—	—	
10 ⁻⁷	5S 1D								
10 ⁻⁸	6S								

S = mouse survived observation period of 11 days.

D = mouse died with ++++ pulmonary consolidation.

In the mouse lung, the slope of the neutralization line is 1.4, indicating that the ratio between virus and antibody is not constant, but changes progressively as the two variables are altered.¹ If the test is adequately controlled this exponential relation does not affect measurements of the difference in antibody concentration of different sera (TABLE 2) even though varying quantities of virus are used.

There is another type of neutralization test that can yield quantitative information of a higher order. This depends on the counting of infective virus units, as distinct from an arbitrary end point, and makes use of either the pock technique on the chorioallantoic membrane⁸ or the plaque technique in monolayer tissue culture.⁹ When, as with vaccinia virus, one pock results from infection of the chorioallantoic membrane with one virus particle,¹⁰ it is possible to examine the kinetics of neutralization with high precision and ultimate sensitivity. The rate of the reaction, the quantitative relations between serum and virus, the effects of environmental conditions, and the influence of the host system can be determined in an unambiguous manner.⁸⁻¹⁰ However, the technical difficulties of either the chorioallantoic pock technique or the monolayer culture plaque technique and the limited number of viruses with which they have been used considerably restrict their wide application and general usefulness.

With some viruses, under special conditions, the end-point test could probably be made nearly as precise and almost as sensitive as the pock or plaque techniques. However, to have significance comparable to measurements based on counts of infective units, the procedure would demand simple proportionality between the amount of virus and the response of the host, a requirement that has not yet been met.

A virus neutralization test has one of four objectives; (1) identification of a virus or an immune serum, (2) measurement of antibody concentration, (3) estimation of antigenic relatedness, or (4) measurement of some parameter of the neutralization reaction.

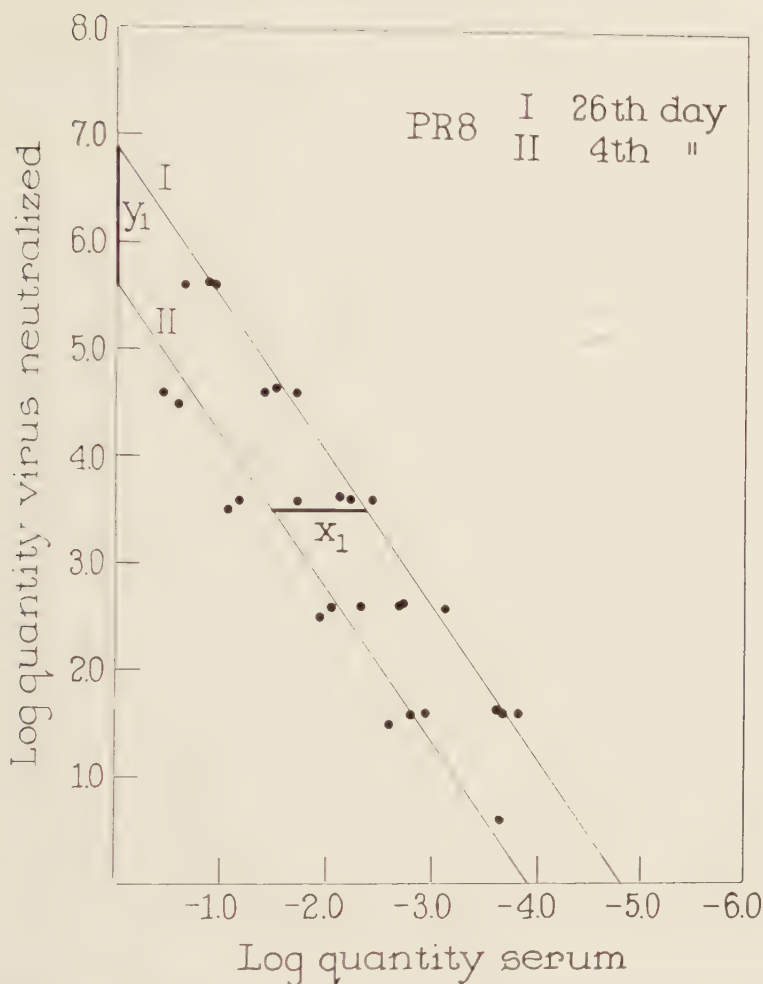


FIGURE 1. Relation between the quantity of immune serum and the quantity of influenza A virus (PR8) neutralized in the lung of the mouse. Acute-phase and convalescent sera from a patient with influenza A.² Reproduced by permission from *The Journal of Experimental Medicine*.

The test can be made extremely sensitive—so delicate, in fact, that it could probably detect a few hundred molecules of antibody⁹ or a very few infective virus particles.^{9, 10} This exquisite sensitivity is one pitfall that can lead to misinterpretation. Thus, minor antigenic components in one virus may lead to antibodies that can neutralize small amounts of another virus only distantly related and suggest similarity.¹¹ More often, however, common antigens may be missed in tests of high sensitivity and distinctness between viruses assumed when relatedness is the fact.¹²

Neutralization is dependent on a number of subtle and complex biological reactions. Not only must host cells be infected and support virus reproduc-

TABLE 2
MEAN 50 PER CENT MORTALITY DILUTION END POINTS OF ACUTE-PHASE AND CONVALESCENT HUMAN SERA AGAINST THE W.S. STRAIN OF INFLUENZA A VIRUS²

Virus		Mean 50 per cent mortality serum dilution end point		Increase in titer
Dilution	Approximate 50 per cent mortality doses	Acute phase	Convalescent	
	log			
10 ⁻¹	10 ^{6.5}	—	1:11	—
10 ⁻²	10 ^{4.5}	1:11	1:87	7.9×
10 ⁻³	10 ^{3.5}	1:59	1:490	8.3×
10 ⁻⁴	10 ^{2.5}	1:302	1:2510	8.3×
10 ⁻⁵	10 ^{1.5}	1:1120	1:9550	8.5×

tion, but also they usually must be damaged sufficiently so that their abnormality is obvious and can be visualized. In most procedures, the presence of infective or unneutralized virus is determined by simple inspection of the host tissue or by adding indicator cells, for example, erythrocytes.

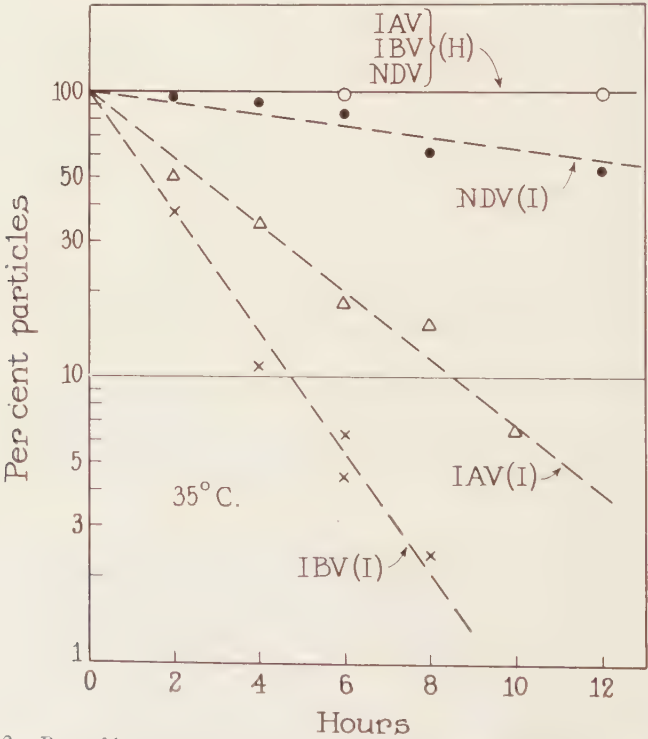


FIGURE 2. Rate of inactivation of infectivity (I) of influenza A (IAV), influenza B (IBV), and Newcastle (NDV) viruses at 35° C. in the allantoic fluid. Hemagglutinating particles - (H).¹³ Reproduced with the permission of *The Journal of Experimental Medicine*.

TABLE 3
NEUTRALIZATION OF NEWCASTLE DISEASE, INFLUENZA A, B, AND MUMPS VIRUSES BY A
HEAT-LABILE COMPONENT OF HUMAN, GUINEA PIG, AND RABBIT SERA¹⁴

Serum	Mean neutralization titers of unheated sera against indicated virus*			
	NDV†	PR8†	Leet	MV‡
Human.....	1:10	Ab.§	Ab.§	1:54
Guinea pig.....	1:19	1:21	1:13	1:48
Rabbit.....	1:13	1:2	1:4	<1:8
Mouse.....	<1:2	—	<1:2	—

* In every instance in which a titer is given the heated serum failed to cause any neutralization.

† 10⁸ E.I.D. employed.

‡ 10² E.I.D. employed.

§ Specific antibodies present.

Many factors other than antibody can affect the reaction and lead to a visual result identical with that produced by specific neutralization. The infective property of a virus is its most subtle and unstable character (FIGURE 2). At 35° C., the half life of an infective particle is in many instances surprisingly brief.¹³ With influenza and mumps viruses it can be less than 2 hours, and with so-called stable viruses, such as Newcastle, poliomyelitis, and vaccinia, it rarely is longer than 24 hours. Consequently, holding virus-serum mixtures in warm environments can affect results unless thermal inactivation is taken into account.

Serum components other than antibody can inactivate some viruses and closely mimic specific neutralization. One such (TABLE 3), the so-called heat-labile inhibitor, present in human serum and that of various experimental animals, inactivates influenza, mumps, and Newcastle viruses as effectively as do homologous antibodies.¹⁴ This inhibitor can be removed by heating serum at 56° C. for 30 minutes. The substance has caused serious difficulty and can lead to the assignment of etiological significance to a virus when, in fact, no such relation exists.

Other serum components, some of which are highly unstable, may augment the neutralizing action of antibody and lead to more inactivation than will antibody itself.⁹ Such "cofactor" activity probably explains the irregularities that have resulted from the addition of fresh normal serum to virus-serum mixtures and the divergent views as to the effect of hemolytic complement on neutralization tests.

The degree of susceptibility of the host also is an important factor.⁷ In exquisitely susceptible hosts, which respond to one or a few virus particles, neutralization must be almost complete if evidence of it is to be seen. On the other hand, in relatively resistant hosts, which respond only to large numbers of particles, a modest reduction in infective particles can produce a large effect on response and suggest that full neutralization has occurred.

The mechanism of virus neutralization by immune serum is not yet fully understood, but the kinetic aspects of the reaction are becoming clearer. They

have been most effectively and precisely studied by either the pock⁸ or the plaque techniques,⁹ which permit counting of infective virus units. Neutralization or inactivation of infectivity by homologous antibody appears to depend on combination of virus particle and antibody molecules. The rate corresponds with a first-order reaction during the early stages. A constant proportion of infective particles is inactivated per unit of time. The rate of inactivation may be rapid, but it depends both on the concentration of antibody and on the temperature. The lower the antibody concentration or the temperature, the slower is the reaction. Apparently, inactivation *in vitro* is never complete, and approximately one infective particle per thousand escapes neutralization and persists. There is evidence indicating that attachment of a single molecule of neutralizing antibody will inactivate a virus particle.⁹

Although a number of possible explanations for the persistent small fraction of unneutralized particles have been pursued, an entirely satisfactory hypothesis has not yet been offered. Apparently, the persistent fraction cannot be attributed to (1) reactivation by dissociation from antibody, (2) absence of cofactors, (3) presence of inhibitors, or (4) heritable differences in the virus particles.⁹ At low antibody concentrations the unneutralized fraction appears to depend on the antibody-virus ratio.

Important as these findings are to understanding the dynamics of inactivation of virus infectivity by antibody *in vitro*, they do not entirely clarify all features of neutralization as determined *in vivo*.⁷ It has been proposed recently⁹ that the presence of antibody together with the unneutralized fraction of virus in the mixtures accounts for certain of the findings with the end-point technique.

The great majority of neutralization tests, whether performed in intact host species or in tissue cultures, utilizes an end-point technique. For numerous technical reasons, procedures of this kind are likely to remain the mainstay for some time. The features of the end-point technique that lead to difficulties of interpretation are (1) effects of diluting the serum, (2) effects of the host system, (3) effects of the route of inoculation, and (4) effects of the nature and amount of virus used.⁷ These are, to a large degree, interdependent, and none can be wholly separated from the others.

The serum-dilution effect is especially striking in the allantoic sac of the chick embryo.⁴ In a chessboard experiment, in which multiple dilutions of both influenza virus and homologous immune serum are used, it is obvious that the more the serum is diluted the less effective it becomes (FIGURE 3). The ratio between the amount of serum and the infective units of virus neutralized is not constant. The slope of the neutralization line¹ which relates the two quantities is steep. In this system a tenfold change in the amount of serum causes a fifty-thousandfold change in the amount of virus neutralized. With the two sera used, one neutralizes more virus or contains more antibody than the other, but how can the difference be expressed numerically?

The neutralizing titer of a serum depends on the amount of virus used and therefore is a relative value. The relation between the two quantities (TABLE 4) is readily expressed, but has an exponential form; that is, $y = bx^a$.¹ In this system the exponent a is very large (4.7). All systems studied have shown an

TABLE 4
RELATION BETWEEN AMOUNTS OF SERUM AND VIRUS WITH DIFFERENT SLOPES
OF NEUTRALIZATION LINE¹

Slope*	Changes in amount	
	Serum	Virus
1.0	10	10
1.5	10	32
2.0	10	100
3.0	10	1,000
4.0	10	10,000

* Slope = exponent a , in $y = bx^a$.¹

TABLE 5
NEUTRALIZING ACTIVITY OF IMMUNE SERA AGAINST INFLUENZA A VIRUS

Value*	Serum 1	Serum 2	Difference
	<i>log</i>	<i>log</i>	<i>log</i>
Titer vs. 10 ³ E.I.D.	-1.75	-2.60	0.85
Dilution vs. 1 E.I.D.	-2.35	-3.20	0.85
Neutralizing capacity	11.21	15.21	4.00

* Neutralization in the allantoic cavity.

exponential relation,⁷ although no other gives an equally high value or has as steep a slope.

The problem of assigning numerical values to a serum can be handled in three ways (TABLE 5): (1) the serum titer against a constant amount of virus (for example, 1000 infective units); (2) the serum dilution by extrapolation (that is, the intercept on the x axis) that will neutralize one infective unit (FIGURE 3); or (3) the maximum number of infective units by extrapolation (that is, the intercept on the y axis), which the undiluted serum will neutralize. The latter value is termed the neutralizing capacity¹ and has certain theoretical advantages if the slope of the neutralization line is 1.0. However, when the slope is steep, as with neutralization in the allantoic cavity, the neutralizing capacity shows greatly expanded differences between immune sera.

The effects of the host system are evident (TABLE 6) on comparison of results with influenza virus in the allantoic cavity of the chick embryo, mouse lung, and chorioallantoic membrane culture.⁷ The susceptibility of these host systems is widely dissimilar and spans a range of about ten-thousandfold. The most susceptible medium, namely, the allantoic cavity, shows the steepest slope; the less susceptible hosts yield gradual slopes. With a constant quantity of virus, but without a constant number of infectious units relative to the host, the most susceptible host gives the lowest serum titer; the less susceptible hosts give higher titers. This is in part a function of the slope of the neutralization line. However, with a constant number of infectious units relative to the host, the relation is reversed; the most susceptible host yields the highest serum titer,

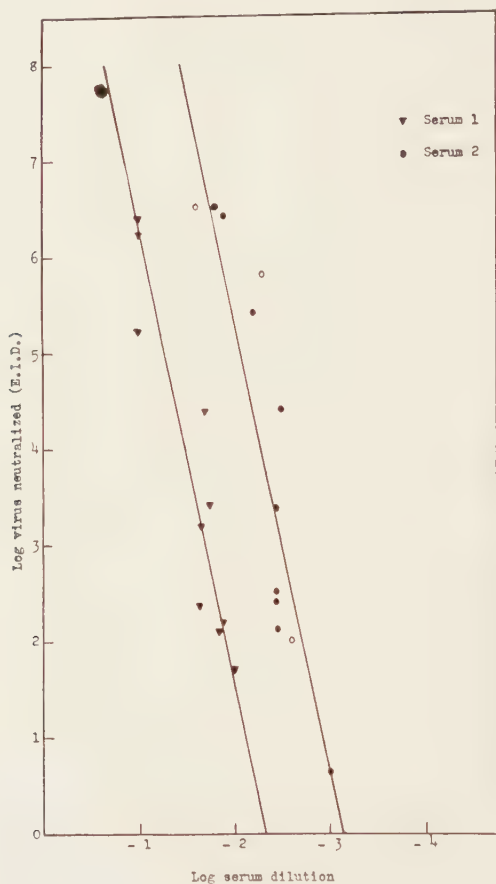


FIGURE 3. Relation between the quantity of immune serum and the quantity of influenza A virus (PR8) neutralized in the allantoic cavity of the chick embryo. Sera from immunized rabbits.⁴ Reproduced by permission from *The Journal of Experimental Medicine*.

and the least susceptible yields the lowest. Other animal species and other tissue-culture systems may show different effects. The foregoing underlines the importance of determining the quantitative relation between virus and serum in any host system that is to be used commonly.

The effects of the route of inoculation (TABLE 7) are apparent when the results of allantoic cavity and chorioallantoic membrane inoculations in the chick embryo, or lung and brain inoculations in the mouse are compared.⁷ In the same host species, susceptibility, the slope of the neutralization line, and serum titer against a constant amount of virus may all vary independently when different routes are used. Even when host susceptibility is taken into account, and the serum titer is determined against a constant number of infectious units relative to route, some spread in the values is seen. There is, as yet, no satisfactory way in which a constant numerical value for the serum can be extracted from results secured under different experimental conditions.

TABLE 6

EFFECTS OF HOST SYSTEMS ON NEUTRALIZING ACTIVITY OF IMMUNE SERA AGAINST INFLUENZA A VIRUS⁷

Host system	Virus titer	Slope of neutralization line	Serum titer versus	
			Constant virus*	Constant infectious units†
	<i>-log</i>		<i>-log</i>	<i>-log</i>
Allantoic cavity	7.5	4.7	1.8	2.0
Mouse lung	4.0	1.4	3.1	1.8
C.A.M. culture	3.7	1.0	2.8	1.1

* Dilution = $10^{-2.5}$.† 10^3 infectious units, relative to host system.

TABLE 7

EFFECTS OF ROUTE OF INOCULATION ON NEUTRALIZING ACTIVITY OF IMMUNE SERA AGAINST INFLUENZA A VIRUS⁷

Host	Route of inoculation	Virus titer	Slope of neutralization line	Serum titer versus	
				Constant virus*	Constant infectious units†
		<i>-log</i>		<i>-log</i>	<i>-log</i>
Chick embryo	C.A.M.	8.5	2.6	0.9	2.1
Chick embryo	Allantoic	7.5	4.7	1.8	2.0
Mouse	Brain	4.5	1.0	2.1	0.9
Mouse	Lung	4.0	1.4	3.1	1.8

* Dilution = $10^{-2.5}$.† 10^3 infectious units, relative to host and route.

The effects of the amount of virus used are, of course, most evident in the exponential character (TABLE 8) of the quantitative relation between virus and serum.^{1, 2, 4, 7} Only in a few host systems has this relation approached one of simple proportions. With influenza in chorioallantoic membrane culture or the mouse brain, a 1:1 relation has been observed.⁷ However, it is not evident that this would occur with other viruses in the same hosts. Influenza and Newcastle viruses give similar exponential slopes (about 2.0) on the chorioallantoic membrane,^{7, 15} and likewise give comparable steep slopes in the allantoic cavity^{4, 7, 16} (about 4.0), as also does mumps virus.¹⁷ Influenza¹ and pneumonia virus of mice (PVM)³ show identical slopes (1.4) in the mouse lung, and this slope is similar to that given Western equine encephalitis (WEE)¹⁸ and herpes simplex⁵ in the mouse brain and peritoneum, respectively. With poliovirus in monkey kidney tissue culture,^{19, 20} it appears that the slope is in the neighborhood of 1.5, as is also the case with mumps virus in HeLa cell cultures.²¹ With herpes simplex in the yolk sac,²² a slope of about 2.0 has been found. Bacteriophage T₃, studied under identical conditions, shows a slope of about 2.0 on *E. coli* B *in vitro*, regardless of the number of unneutralized particles taken as the end point.⁷

TABLE 8
QUANTITATIVE RELATION BETWEEN AMOUNTS OF VIRUS AND SERUM IN
NEUTRALIZATION EXPERIMENTS

Host	Slope of neutralization line							
	Influenza	NDV	Mumps	Herpes simplex	PVM	WEE	Polio	T.
Mouse, lung	1.4				1.4			
Mouse, brain	1.0					1.4		
Mouse, intraperitoneally				1.4				
Chick embryo, chorioallantoic membrane	2.1	1.6						
Chick embryo, allantoic cavity	4.7	4.2	3.2					
Chick embryo, yolk sac				2.0				
Culture, chorioallantoic membrane	1.0							
Culture, monkey kidney							1.5	
Culture, HeLa			1.5					
<i>E. Coli</i> B.								2.1

For the investigator concerned with the measurement of antibody concentration, the practical difficulties introduced by the effects of slope of the neutralization line, host susceptibility, route of inoculation, and amount of virus used are these:

Neutralizing antibodies may be found with one set of conditions, but not with another. A high neutralizing titer may emerge under some conditions and a low titer under others. With two sera from the same individual, a large increase in the amount of antibody may be indicated under some conditions and a small increase under other conditions. Comparison of antibody levels measured under different experimental conditions is not feasible without extensive and precise information about the effects of all the variables used.

Many of the uncertainties in interpretation can be eliminated by careful control, standardization, and quantitation of the neutralization test. A satisfactory test should be sensitive, precise, and reproducible and, if possible, it should have a percentile end point. The slope of the neutralization line (that is the relation between the amounts of serum and virus) should be carefully determined, and the information used to the fullest extent. The susceptibility of the host system should be measured frequently and maintained as nearly constant as possible. The route of inoculation should not be varied. The amount of virus used should be nearly constant as measured by titration in the same host system. It should not be so large as to override small amounts of antibody, nor so small as to be overcome by components other than antibody or environmental effects. The constant virus-serum dilution technique has numerous advantages,⁷ both theoretical and practical, and it directly reflects differences in antibody concentration even in host systems with the steepest slopes.¹ Multiple serum specimens from one individual should be examined simultaneously whenever possible, and stored frozen at a temperature lower than -15°C . between tests.

Important and informative though the neutralization test is in studies on virus infections and diseases, it should be supplanted whenever possible by an *in vitro* test if this will yield equivalent information.

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RADIOLABELED ANTIBODIES

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Radiolabeled antibodies are especially useful in cancer studies for determining what happens to antitumor or antitissue antibodies when they are injected *in vivo* and in determining whether or not such antibodies localize in the tumor or tissue against which they were formed.¹ Actually, in the case of antitumor antibodies there is a possibility of using such radiolabeled antibodies diagnostically as indicators to determine the locations of tumors and their metastases and, perhaps, even therapeutically to carry physiologically active concentrations of radioactivity to the tumor.²⁻⁴

In this article, the use of radiolabeled antibodies in the study of cancer will be discussed, and emphasis will be placed on pitfalls that are known to be present.

Methods of radiolabeling antibodies

Antibodies may be radiolabeled in two different ways: *in vivo* by incorporation of the label in the antibody molecule as it is formed, or *in vitro* by coupling a radioactive substance to the antibody already formed. The *in vivo* method is carried out by feeding radioactive amino acids or precursors of amino acids to the animal or cells producing antibodies so that the proteins, (that is, the antibodies) formed by the animal or cells in culture are naturally labeled. Radioactive carbon and sulfur have been incorporated. Naturally labeled antibodies suffer from the disadvantages that only low levels of radioactivity are incorporated, and that there is always the problem of reincorporating the labeled material into the body following metabolism when used for *in vivo* localization experiments. The *in vitro* method, in which a radioactive substance is coupled to antibody already formed, has the following advantages: (1) much higher levels of radioactivity can be incorporated; (2) the labeling is much simpler to achieve; (3) radioactive materials that are measured more easily than carbon or sulfur can be used; and (4) for *in vivo* localization experiments, labels that are not reincorporated into the animal's own proteins after metabolism can be chosen. Substances so far reported for use as coupled radioactive labels are iodine,⁵⁻⁶ which has been incorporated by direct iodination of the protein, and sulfur, which has been incorporated as *p*-azobenzene sulfonate groups.⁷ Of course, there is a host of substances that can possibly be used as radioactive labels; among them are those reported for labeling antigens in some immunological studies.⁸ Another label that has not been reported heretofore and that we have found to be very useful is the *p*-iodobenzoyl group label.⁹

It is important that the coupled group should not destroy antibody. Coupling antibody with small amounts of the reagents mentioned above (one or two groups per antibody molecule) produces only a small effect, if any, on the various reactions of antibody such as precipitation, localization, ability to adhere to solid antigen, and ability to produce hemolysis.⁹⁻¹¹ Coupling

larger amounts does decrease antibody activity, although quite a large amount can be coupled (over 50 iodine atoms per molecule) without destroying the antibody activity completely.¹⁰

Stability of the bond between the antibody and the label is very important, especially in *in vivo* studies. The iodine label, which has been studied extensively, remains attached *in vivo* over long periods of time. For example, Melcher and Masouredis showed that iodinated antihuman serum albumin antibodies injected into a guinea pig are stable. The iodine/antibody ratio remained constant until the guinea pig began to produce antibodies against the injected rabbit protein.¹² Stability of the iodine protein bond *in vivo* has also been demonstrated in the case of localized antibodies. The iodine-antibody bond in kidney-localized antikidney antibody has a half life of at least twenty days.¹³

In using a protein with many groups coupled to it, one must remember that the various individual molecules contain different numbers of coupled groups. In a system of antibody molecules containing an average of several coupled groups the distribution is such that some of the labeled antibody molecules will contain only one coupled group, whereas others will contain many more than the average. One must be careful, then, that he is not studying the selected action of the more highly coupled material. The safest procedure is to couple so that there is, on an average, less than one group per antibody molecule. It is then safe to assume that whatever antibody is being followed is an antibody that contains only a single coupled group per molecule and thus has undergone minimum alteration.

In order to couple the label efficiently with antibody, the gamma-globulin fraction is separated from the other serum components. It is important to remove the albumin, especially if coupling is by iodination or diazo coupling, since albumin couples in these reactions much more rapidly than does globulin.¹⁰ Crude globulin fractions contain components that iodinate at a rate greater than that of the antibody itself. Antibodies of different activities and specificities appear to iodinate at the same rate.¹⁰ If the gamma globulin fraction is carefully prepared and is free of albumin and other globulins, as is most readily achieved when there is a large proportion of antibody globulin present, the iodination of the antibody proceeds at a rate equivalent to that of the remainder of the globulin fraction.^{11, 14} Indeed, in quantitative precipitin reactions with carefully prepared material, the fraction of radioactivity precipitated by antigen is the same as the fraction of the protein present that is precipitated.¹¹

Specific Purification of Labeled Antibody

In order to obtain labeled antibody with the highest portion of label attached to the antibody of interest, it is necessary to resort to specific purification of the antibody itself. This can be achieved by labeling antibodies specifically purified by methods described in the literature, or else by purifying antibody already labeled. The latter technique is accomplished by absorption of the antibody on the solid-specific absorbant, removing the supernate, and then by eluting the specifically absorbed antibodies by one of the four following meth-

ods: (1) by changing the pH;¹⁵ (2) by changing the temperature;¹⁶ (3) by adding substances active in breaking hydrogen bonds, such as urea or salicylate;¹⁷ or (4) by eluting through exchange with uncoupled antibody.¹⁸ The first methods of elution listed may run the risk of altering or denaturing the antibody somewhat, but this has been proved not to occur to a particularly detrimental extent, since there is obtained a labeled product with definite localizing specificity which can be evaluated. Moreover, the antibody thus obtained can be recoupled with more radioactive label to yield a product of high specific radioactivity that contains a minimum of radioactivity associated with substances other than specific antibody. The exchange method probably yields a less altered antibody. However, it suffers from two disadvantages: namely, the fact that the presence of the large amount of unlabeled nonantibody protein in the product does not permit enhancing the radioactivity content of the specific antibody by recoupling, and the further fact that added unlabeled antibody can compete for antigen sites with labeled antibody in subsequent reactions.

Tumor-Localizing Antibodies

In studies of tumor-localizing antibodies, the problem of specific purification is of major importance. When antibodies are produced against a homogenate of a solid tumor, there are also formed cross-localizing antibodies that will localize in normal tissues as well.^{4, 19-22}

It is important to increase the concentration of tumor-localizing antibodies and decrease the concentration of the cross-localizing antibodies. Concentration of the localizing antibody can be accomplished by absorbing the antibody on the tissue sediment and subsequently eluting it.¹⁹ When whole tumor tissue sediment is used, however, this results in a concentration of both tumor and cross-localizing antibody. Some increase in specificity can be obtained by first absorbing out cross-localizing antibody with normal tissues.²⁰

Another way of reducing cross-localization is to use purified antigens for the production of antibody or for purification. This has been accomplished in our laboratory²⁰ by using ascites cells derived from the tumor of interest, namely, the Murphy lymphosarcoma. The purification is described elsewhere in these pages by Eugene Day and myself.

In the preparation of localizing antibodies it is important to remember that while there is a multitude of antigenic substances in the tumor, many of these cannot be effective *in vivo* in fixing the corresponding antibody due to their position in the cell. This has been shown clearly in the case of the kidney. Antibodies that precipitate with the soluble portions of kidney are not important for localization, since they apparently cannot reach their corresponding antigen *in vivo*,²³ in order to combine with it. In our work, we have found that antibody capable of localizing in normal tissues and tumors can be formed against the saline insoluble fraction of the tissues. We believe that this fraction is important, since it contains cell-surface material, and that it is the cell surface on which localization of antibody takes place.

Failure to obtain localizing antibodies by injecting a particular tissue fraction does not mean that localizing antibodies cannot be formed against the

particular tumor, but may be due to the use of the wrong fraction for immunization. This explanation has been used by Mason *et al.*,²⁴ who observed that antibody against the melanoma granules of the Harding-Passey melanoma do not localize in the tumor. These investigators postulated that the antibody could not penetrate *in vivo* into the cell to reach the granules.

Nonspecific Localization in Tumor

Another problem of major importance in the study of tumor-localizing antibodies is the nonspecific localization of proteins in general. Tumor has the property of picking up foreign substances nonspecifically.²⁵ This appears to be due to capillary permeability of tumor blood vessels.

This nonspecific background is so great that it has been used diagnostically for determining the location of tumor tissue.^{26, 27} However, the nonspecific localization is the background localization above which must be observed any localization due to localizing antibody. This greatly increases the difficulty of determining the concentration of localizing antibody present in a preparation containing only a small amount of such antibody. In the case of animal experiments (for example, experiments with mouse tumors or rat tumors) it is possible to determine the background localization of control globulin in several tumor-bearing animals. Localization from a labeled antibody preparation above this background in another group of tumor-bearing animals is interpreted as antibody localization. In the investigation of the localization of antibodies in human tumors, it is not possible to obtain a large series of individuals with the identical tumors so that some can be injected with control protein, and others with antitumor-antibody protein. Neither would it be good practice to inject an individual first with control protein, assay the localization in a specimen removed by biopsy, and then inject antitumor antibody and determine the localization of this material in another specimen; since localization in a tumor is not uniform, the control thus would not be valid.⁹ Moreover, the individual may produce antibodies affecting localization between successive injections.

In order to circumvent this difficulty in controlling the experiment, we have developed a system of paired labels for such investigations. A paired label permits the control material to be labeled with one radioactive substance and the antiserum to be labeled with another. Both are injected simultaneously, and any pickup of the tumor-localizing antibody can be determined by the differential uptake of the two labels. There is the problem, however, that one labeled protein may be picked up nonspecifically somewhat differently from another labeled protein because of chemical differences in the label or treatment. This was the situation when the pickup of I^{131} -labeled globulin was compared with the pickup of S^{35} -labeled globulin.⁷ Therefore, we decided to use the same chemical label for the two proteins with the labels differing only isotopically. We have developed two types of paired labels; one involves iodination with I^{131} , and either I^{133} or I^{124} as the other member of the pair; the other involves *p*-iodobenzoylation with carbon and iodine-labeled iodobenzoylchloride as members of the pair.⁹

An assay of the concentration of two of the paired labels is carried out as

follows: in the case of the iodine labels, a tissue sample is counted in a well-type scintillation counter equipped with a two-peak analyzer. Since the iodines have different gamma-ray spectra, they can be determined in the presence of each other. In the case of the *p*-iodobenzoyl labels, the sample is prepared by hydrolyzing the tissue in sodium hydroxide, adding carrier *p*-iodobenzoic acid, and acidifying and extracting the *p*-iodobenzoic acid into ether. The ether solution is added to a suitable liquid phosphor, and both labels are counted individually in a dual-channel liquid phosphor counter.

Nonuniform Localization

Another problem that arises with tumor-localizing antibodies is that of non-uniform distribution of radioactivity in the tumor as described above. When an animal is injected with radiolabeled protein, the tumor does not pick up the protein or antibody uniformly, but there seem to be some volumes which show greater concentration than others. This problem can be handled very well by the use of paired labels, since the relative localization of specific antibody and control protein can be determined. The interesting result has been that the localization of antibody or control protein is neither uniform nor parallel.⁹ The concentration of antibody is much more variable, and probably reflects different rates of blood flow through different parts of the tumor.

Another factor contributing to nonuniform localization is growth of tumor during assay period, but the paired label is effective in controlling this factor.

Rate of Localization in Tumors

Another factor that is important in determining the localization of antitumor antibodies is the slow rate of localization in tumors. We know that in order for an antibody to localize in an organ or a tissue, contact must be made between the antibody and the antigen site of localization. Moreover, most rapid localization would be that which takes place directly from the blood-stream. Some organs, including the kidneys and the liver, are known to clear the blood of certain of their respectively localizing antibodies as the blood passes through the organ.^{28, 29} As a result, they can clear the blood to the extent of 95 per cent of this localizing antibody within 15 minutes because of the high rate of blood flow through them. Even if tumors localized antibody directly from the blood stream, the rate of clearance of tumor-localizing antibody from the blood would be much lower, limited by the much smaller blood flow through the tumor. If the rate of flow of blood through a tumor were only 1 per cent of that through the kidney, about 25 hours would be required to clear the blood to the extent of 95 per cent, and 6 hours for clearance to the extent of 50 per cent. Wissler has remarked that antibody localization in the Flexner-Jobling tumor increases with time through the second day.²²

Summary

In summary are listed several important pitfalls to avoid in studying localizing antitumor antibodies: (1) failure to use proper control and to take into account nonspecific localization; (2) failure to concentrate specific tumor-lo-

calizing antibody and remove cross-localizing antibody; (3) failure to prepare antibody against proper tissue components; (4) failure to take into account the slow rate of localization in tumor; (5) failure to take into account nonuniform localization; and (6) failure to use proper label or to label properly.

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PURIFICATION OF TUMOR-LOCALIZING ANTIBODIES*

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We propose in this paper to show how we have been able to obtain antitumor antiserum fractions that assay as high as 30 per cent for tumor-localizing antibodies. The tumor-host combination used for this study was the solid-ascites form of the Murphy lymphosarcoma in Wistar rats. Antisera were prepared in rabbits against both the solid-ascites and free-ascites forms of the lymphosarcoma. We isolated gamma globulins from these sera by salt fractionation and labeled them at the beginning of each experiment with I^{131} (about 1 mc./10 mg. globulin) plus enough nonradioactive carrier iodine to yield a maximum average of 1 iodine atom per globulin molecule.†

In order to determine whether an antiserum globulin contains any tumor-localizing antibodies, we have always found it necessary first to concentrate the antibodies from the globulin. The reason for this is that the solid lymphosarcoma picks up a relatively large amount of material nonspecifically from the intravenously injected protein. This high nonspecific localization of background proteins masks the localization of proportionately small amounts of antitumor antibodies. However, since this localization background increases directly with the size of the tumor and inversely with the size of the rat (FIGURE 1), it has been possible to derive a formula for the estimation of background localizations of tumors subsequently used for assays of concentrated tumor-localizing antibodies. The total observed localization of radiolabeled globulin in any of these tumors, minus the calculated value, gives a net localization that has been equated to true tumor localization. In normal tissues the background localizations are of a lower order and are less variable from rat to rat. Liver contains, on the average, 1.2 per cent of the injected dose after one day, whereas a tumor of equivalent weight (about 5 per cent of the body weight) contains four times this amount. Both kidneys contain 0.19 per cent; spleen contains 0.13 per cent. Antibody localization in these normal tissues has been equated, therefore, to net localization obtained by subtracting the above background values from the total observed localization for these tissues.

To assay an antiserum for the presence of tumor-localizing antibodies, we have been using a single elution procedure described briefly in TABLE 1. The antiserum globulin and tumor sediment are shaken together for one hour at 37° C. and centrifuged. The sediment containing the absorbed antibody is then washed thoroughly and suspended in heated saline (60° C., pH 8) where it is shaken for 15 minutes. A portion of the absorbed globulin (35 to 45 per cent) is eluted and separated from the sediment by centrifugation. In a double elution process, this eluate is reabsorbed and re-eluted. The final eluate is

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† The techniques for tumor maintenance, antiserum production, salt fractionation of gamma globulin, preparation of tissue sediments, radioiodination, radioactivity counting, absorption elution procedures, and *in vivo* assay have been described in detail elsewhere.¹

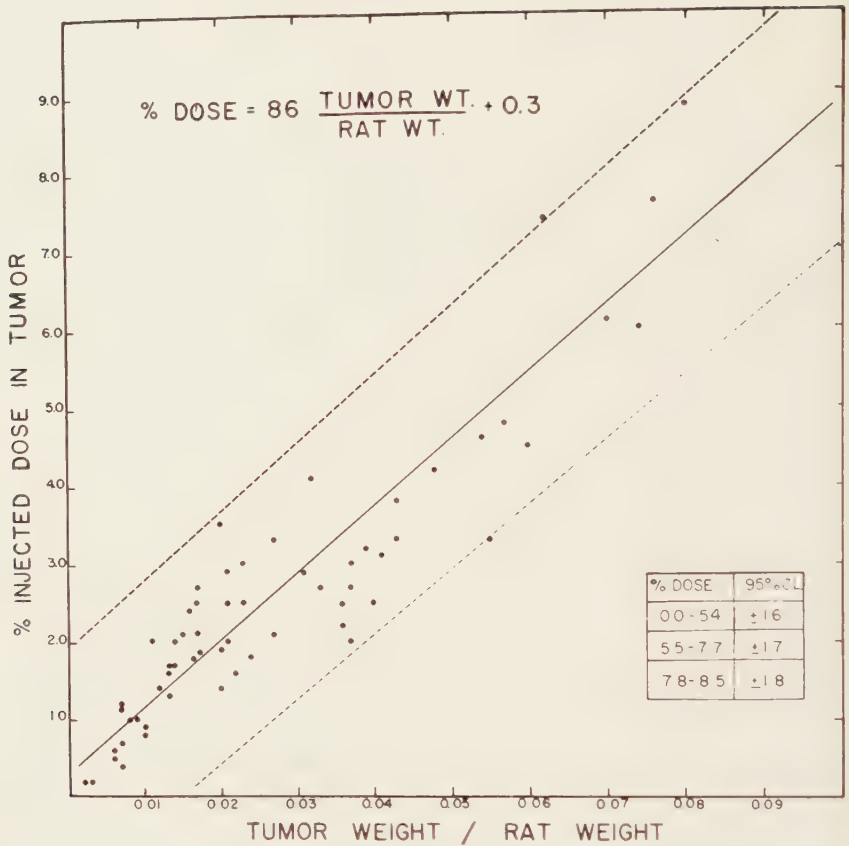


FIGURE 1. The accumulation of radioiodinated nonlocalizing rabbit-serum globulin in the solid-ascites Murphy lymphosarcoma of Wistar rats as a function of tumor size and body size. The solid line is the plot of the least square equation; the broken lines are the hyperbolic 95 per cent confidence limits about the per cent dose; the table shows these limits for a range of values. Reproduced by permission from *The Journal of the National Cancer Institute*.¹

TABLE 1
IN VITRO PURIFICATION OF TUMOR-LOCALIZING ANTIBODIES

Purification process	Steps used, starting with 10 mg. whole globulin labeled with 1 mc. I ¹³¹
Normal tissue absorption	Absorbed with 100 mg. normal tissue sediment. Supernate treated as in double elution.
Double elution	Absorbed and eluted from 100 mg. tumor sediment. Eluate treated as in single elution.
Single elution	Absorbed and eluted from 10 mg. tumor sediment. Eluate treated as in no purification.
No purification	Diluted to desired level of radioactivity with saline. 2-ml. portions assayed in rats for localization properties.

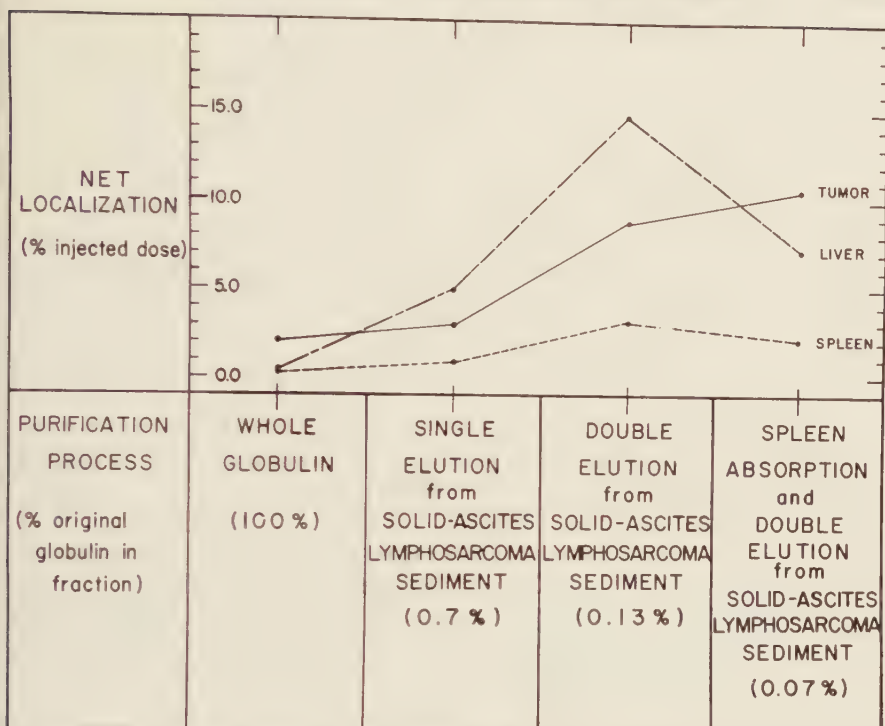


FIGURE 2. *In vivo* localization of antisolid-ascites tumor antibodies purified from solid-ascites lymphosarcoma sediment. See TABLE 1 for an outline of the purification processes used.

injected into tumor-bearing rats and assayed one day later for its distribution in tissues by radioactivity counting procedures.*

In FIGURE 2 is shown a typical assay of antibodies from an antisolid-tumor antiserum that have been purified by single and double elution processes from the sediment of the solid tumor. Liver and spleen localizations, as well as tumor localization, increased threefold as the purification process was carried from a single to a double elution.

How can we tell if the localization in tumor is brought about by antibodies different from those that cross-localize in the normal tissues? From the assays of the singly and doubly eluted materials one easily might have concluded that here, perhaps, there was involved a single type of antibody that distributed itself proportionally among tumor and normal tissues regardless of the extent of purification. The first hint that two types of antibody are indeed involved stems from previous experiments² in which it was shown that antinormal-tissue antibodies did not localize in tumor. The second and more substantial evidence is demonstrated at the far right of FIGURE 2. Here the initial globulin was absorbed, prior to antibody concentration, with sediment from a normal

* See second footnote, page 651.

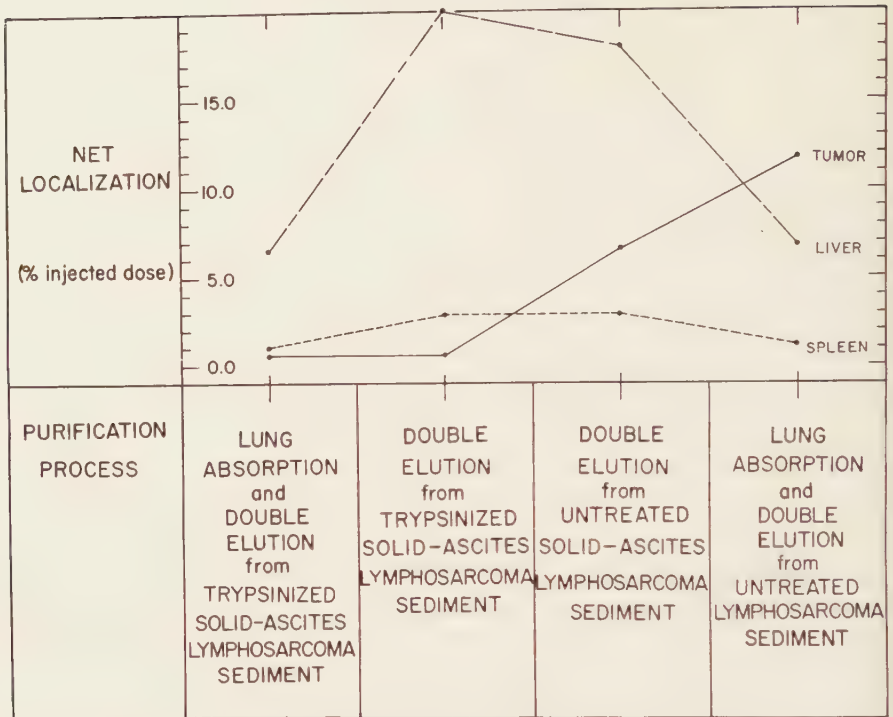


FIGURE 3. *In vivo* localization of antisolid-ascites tumor antibodies purified from trypsinized and untreated solid-ascites lymphosarcoma sediment. See TABLE 1 for outline of purification processes used.

tissue (TABLE 1). The unabsorbed antibodies, when concentrated by double elution from solid tumor, localized to an even greater extent than when processed by double elution alone, whereas they localized in normal tissues, represented here by liver and spleen, to a much lesser extent.

The difference between tumor- and cross-localizing antibodies was further explored in the next experiment (FIGURE 3) where the solid-tumor sediment was trypsinized* prior to its use as an absorption and elution medium. Trypsin, it was found, destroys the antigens in the solid Murphy lymphosarcoma with which tumor-localizing antibodies react, whereas it leaves unimpaired the antigens upon which cross-localizing antibodies are absorbed. Consequently, trypsinized solid tumor serves as a medium for purification of cross-localizing antibodies. These cross-localizing antibodies, incidentally, do not localize detectably in the tumor, even though their localization is as high as 20 per cent

* In this experiment 500 mg. of lyophilized solid-ascites tumor sediment and 50 mg. of crystalline trypsin (Worthington) were homogenized in 500 ml. of 0.9 per cent NaCl that had been buffered to pH 8 with borate. The suspended sediment was incubated under constant agitation at 37° C. for 4 hr., centrifuged, washed twice with 500 ml. portions of buffered saline, relyophilized, and then used for the purification processes indicated in FIGURE 3 and outlined in TABLE 1.

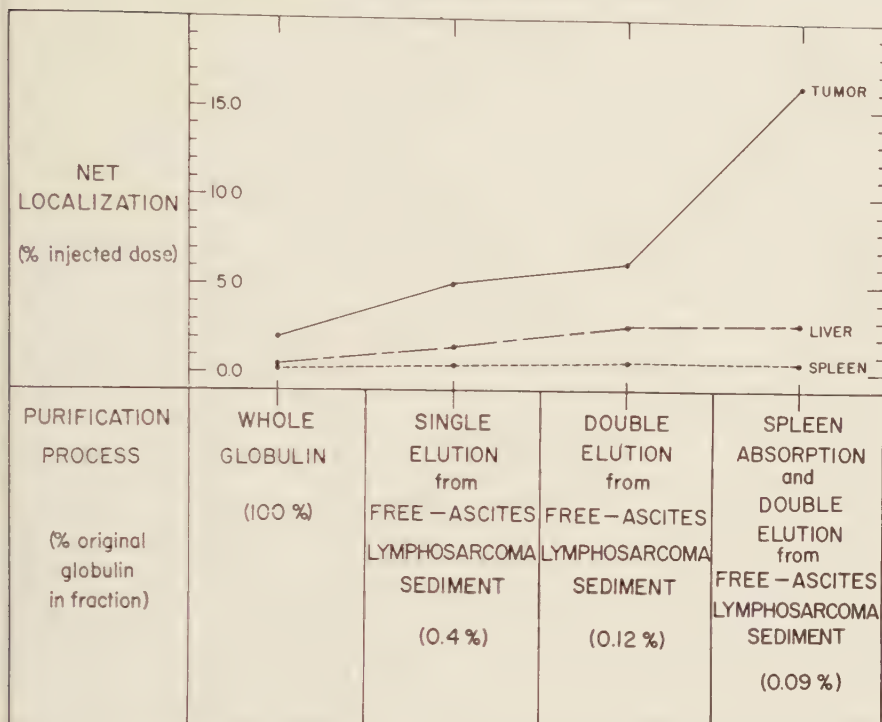


FIGURE 4. *In vivo* localization of antisolid-ascites tumor antibodies purified from free-ascites lymphosarcoma sediment. Net localization in tumor after double elution should read 11 per cent. See TABLE 1 for outline of purification processes used.

in the liver. Indeed, their localization properties greatly resemble those of antinormal-tissue antibodies.

This last procedure was not, of course, very practicable, since we were interested in purifying tumor-localizing antibodies, not those that localize in normal tissues. In other words, we wished to obtain tumor material in which normal tissue components, such as connective tissues and blood vessels, were absent. The free-ascites form of the Murphy lymphosarcoma met this criterion. The extent of purification of tumor-localizing antibodies from an antisolid-tumor antiserum (the same serum as that used for the experiment depicted in FIGURE 2), through the use of sediment from free-ascites cells, is shown in FIGURE 4. As can be seen, tumor-localizing antibodies were purified to a much greater extent by absorption on and elution from the free-ascites tumor sediment than by a similar process utilizing the solid form of the tumor. Furthermore, this purification was at the expense of cross-localizing antibodies that localized, even after double elution from ascites sediment, to the extent of only 3 per cent in liver, in contrast to 15 per cent after double elution from solid sediment.

Having obtained these results with antisolid-tumor antisera, we rather suspected that antisera prepared against the ascites tumor would contain a much greater proportion of specific tumor-localizing antibodies than of the cross-

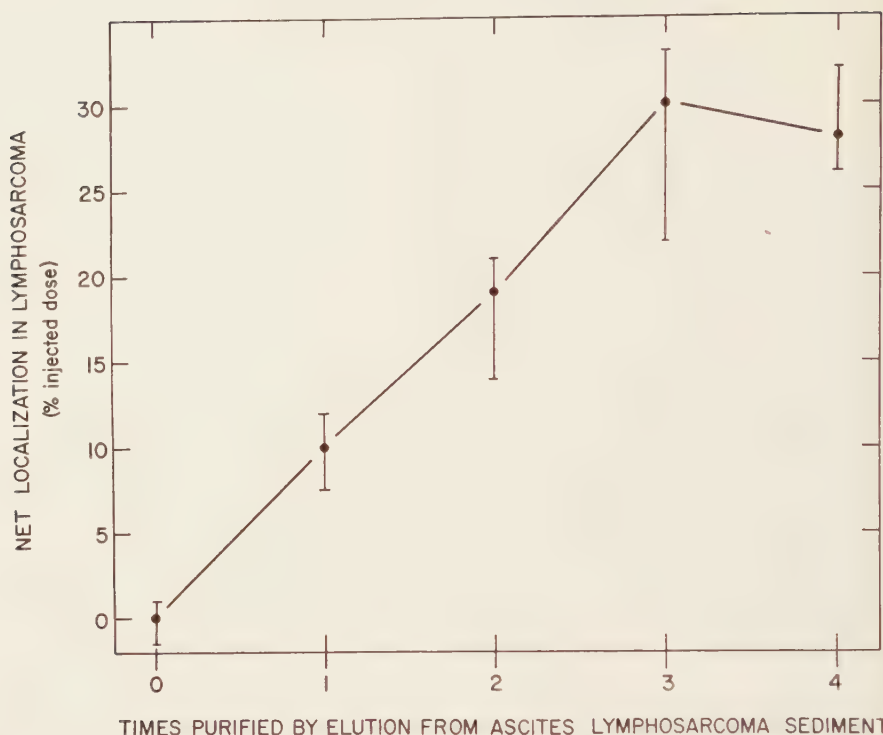


FIGURE 5. *In vivo* localization of antifree-ascites tumor antibodies purified from free ascites lymphosarcoma sediment by serial elution processes (see footnote to text).

localizing type. Moreover, the purification of antibodies from such sera by serial elution from ascites tumor sediment would give us, by the same token, preparations with a high localization potential for tumor. We have previously reported results obtained by the double elution process,¹ but have now extended the purification process through six successive absorptions and elutions from ascites tumor sediment.* Beyond the triple elution step there was no further concentration of tumor-localizing antibodies (FIGURE 5), although there was loss in total material at each additional step. Cross-localizing antibodies were concentrated to a very limited extent by the first elution step, but beyond this point were not concentrated further.†

Thirty per cent net localization in the lymphosarcoma is the highest value we have been able to obtain to date with the methods outlined here. To obtain further purification of tumor-localizing antibodies, we have a number of possibilities: subfractionation of the ascites tumor to obtain cell-surface mate-

* Serial absorptions were performed as follows: 10 mg. of globulin from an anti-ascites-lymphosarcoma antiserum were absorbed by 60 mg. of ascites tumor sediment; the eluate from this was absorbed by 30 mg. of sediment; the second eluate was absorbed by 15 mg.; the third, by 8 mg.; the fourth, by 4 mg.; and the fifth, by 2 mg.

† Net localizations never exceeded 1.0 per cent for liver, 0.2 per cent for kidney, and 0.4 per cent for spleen.

rial from which to prepare and purify antibody is one of these. However, since our criterion of the purity of tumor-localizing antibodies is the extent to which they localize in a tumor and cross-localize in normal tissues of a living rat, we shall have to consider not only additional methods for concentrating antibody *in vitro*, but also the mechanisms underlying localization *in vivo*.

For example, we have accumulated enough data to indicate that the rate of localization in tumor is very slow. As yet we have not measured this rate precisely, but we do know that it takes at least twelve hours and perhaps more for the lymphosarcoma to localize one half of the available antibody. On the other hand, as we have shown previously,³ it takes only a few minutes for the kidney, liver, and spleen to localize their respective antibodies. This difference in rates of localization by tumor and by normal tissues has, in one way, simplified our task. Cross-localizing antibodies, because they are removed from the circulation by normal tissues before reaching the tumor, and specific tumor-localizing antibodies, because they appear to be the only localizing antibodies that do reach the tumor eventually—these two types of localizing antibodies are measurable as single and separate entities even before either is separated from the other.

However, because of this slow rate of localization, it is probable that we have not been measuring all potentially tumor-localizing antibodies when we assay after just one day. If we were to extend the assay time to a second or third day, assuming that continued tumor growth would present no problem, we should still be faced with the continuous metabolism of unlocalized antibody—potentially tumor-localizing, but as yet still circulating—before it has a chance to reach the tumor. In spite of these limitations, the values shown in FIGURE 5 do measure the increased purification of tumor-localizing antibody at least to the extent of 30 per cent and, as such, they do mean that this level is a minimal one that can be obtained with our present methods.

Acknowledgment

The constant and invaluable assistance of Jakob A. Planinsek and his staff of technicians, Mary McMahon, Louis Kubai, and Donald Smith, contributed in large part to the success of our experiments.

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THE APPLICATION OF FLUORESCENT ANTIBODIES TO THE STUDY OF NATURALLY OCCURRING ANTIBODIES

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My role in this monograph is to discuss the use of antibody labeled with fluorescein as a method for the histological localization of antigenic material. I do this with some diffidence because I have no personal work to report and no personal experience with the localization of naturally occurring antigenic substances. However, I hope the reader will find it useful if I review the few instances so far reported in which immunohistochemistry has been applied to the problems involved.

An inescapable condition for the employment of immunological methods is the recognition and acceptance of the fundamentally circular character of the situation. The injection of a mixture of substances of unknown composition or variety results in the synthesis of antibodies specifically reactive with *some* of them. It is this circular dependence on induced biosynthesis that invariably amuses students first exposed to the fundamental definitions of immunology, and that accounts for the long lag that intervened between the discovery of the basic immunological reactions and the isolation and characterization of the antigenic materials responsible for them. Even in the case of bacterial antigens this process is only now well under way. The exquisite specificity of the immune mechanism favors the discovery and differentiation of new substances, but the discovery is essentially a blind one. This logical posture attains its full awkwardness in the study of naturally occurring components of tissues. Fortunately, there are paths out of this circle, and it is a few examples of these that I shall discuss.

Marshall¹ made a study of the localization of adrenocorticotrophic hormone (ACTH) in the pituitary of the hog. For this purpose he prepared antiserum in adrenalectomized rabbits by the injection of alum-precipitated ACTH of standard potency. This material had a potency 120 times less than another highly active ACTH preparation that was available to him in small quantities. Consequently the resulting antiserum must have contained a variety of antibodies against pituitary components other than the hormone sought. After conjugating this antibody solution with fluorescein and using it as a specific fluorochrome on freeze-dried sections of hog, sheep, and beef pituitary and on hog kidney, he found brilliant staining of certain cells of the anterior pituitary of the hog. There were no cells stained in the sheep or beef pituitary or in the hog kidney, although there was strong fluorescence of plasma proteins in the vessels in both hog and sheep tissues. A control conjugate prepared with the serum of a normal rabbit produced weak traces of yellow-green fluorescence in parallel sections. These traces were in the blood vessels, but they did not involve the parenchymal cells in any case. He was able to remove the reaction with the plasma proteins by the addition of small amounts of normal hog serum to aliquots of the anti-ACTH conjugate without affecting the brilliant granular staining in the cytoplasm of the hog pituitary. Sheep pituitary did not react.

Marshall tested the antiserum by adding 50 and 200 $\mu\text{g.}$ of a highly potent preparation of ACTH to 0.5 ml. aliquots of the labeled antiserum. These additions resulted in a precipitate in each tube containing 45 and 66 $\mu\text{g.}$ of total protein, respectively. On bioassay of the supernatant solutions for residual ACTH, roughly one half of the ACTH activity was found to have been removed from the specific conjugate as compared with a similar addition to the conjugate prepared from normal serum. Unfortunately, this investigator did not attempt to stain a section of hog pituitary with this absorbed supernatant.

Marshall reasoned that "because the hormone was shown to react with the antibody, because the tissue was known to contain the hormone, and because in the tissue only the basophiles bound antibody, it appears that the basophiles must contain the adrenocorticotrophic hormone. . . . Multiple antigens in the preparation used for antiserum production could in this instance be detected by the results of tissue staining. The antibodies corresponding to serum protein impurities in the original antigen could be differentially absorbed. Since it is likely that almost all hormones and enzymes isolated from tissues will prove impure by immunochemical if not by the more commonly used physical criteria, it is important to note that this is not an insuperable barrier to localization by the antibody method."¹

As far as it goes, this evidence is consistent with the localization of ACTH. It could perhaps have been refined further by absorbing aliquots of the conjugate with varying amounts of different hog pituitary preparations of varying and known ACTH content. If the amount of added material just sufficient to abolish the staining reaction paralleled the ACTH content of the pituitary powders used for absorption, the evidence would be still stronger. Of course, even here it is possible that material that was not biologically active might still be antigenically active, in analogy with the relation between toxin and toxoid.

Hill and Cruickshank²⁻³ faced a similar situation in the study of the antigenic localization of the antigen responsible for nephrotoxic nephritis. Antisera prepared in rabbits against rat kidney, isolated rat glomeruli, and rat lung all reacted (after conjugation with fluorescein) with rat kidney glomerular basement membrane and tubular basement membrane. The antikidney serum reacted also with the cytoplasm of the renal tubules. The antiglomerulus serum (which did not react with tubular cytoplasm) induced nephritis. Hill and Cruickshank found that their conjugates would react with the basement membrane and reticulum in many rat organs and with sarcolemma and neurolemma, but not with collagen. These labeled conjugates failed to react with tissue sections of guinea pig, pig, rabbit, and human kidney, although staining similar to that in the rat was observed in the mouse kidney. This cross reaction between rat and mouse kidney was reported earlier by Pressman.⁴ Such localization of an antigen capable of inducing nephrotoxic serum nephritis in rats has recently been confirmed and extended by Baxter and Goodman.⁵

In this instance, the sharp localization of an antigenic material, coupled with the biological activity of its antiserum, allowed its visualization in rather wide-

spread distribution, despite the heterogeneity of the material used for immunization.

In another investigation of normal tissue components, Marshall⁶ studied the antigenicity of the zymogen granules in the pancreas. For this purpose he prepared antiserum against crystallized preparations of bovine chymotrypsinogen, carboxypeptidase, deoxyribonuclease, and ribonuclease. Employing thin (1 to 2 μ) freeze-dried sections, he was able to show that chymotrypsinogen and procarboxypeptidase occurred together in each acinar cell, and that all the zymogen granules appeared to contain both enzymes. They were located in the apex of the cell, and the granules themselves as well as the clear cytoplasmic matrix around them appeared brilliantly "stained" by the labeled antibody solutions. The nuclei, the mitochondria, and the basilar cytoplasm did not react. In the case of the depolymerases the localization was more complex. In both cases, the antigen solutions used to stimulate formation of antibody were not pure, so that it was not possible to determine the exact localization of these enzymes in the pancreatic acinar cells. The results obtained showed fluorescent staining of both zymogen granules and the cytoplasm of some of the cells. "Examination of adjacent sections revealed that ribonuclease and deoxyribonuclease antisera stained the cytoplasm of different sets of cells. The nuclei and the mitochondria were not stained."

Finally, Finck, Holtzer, and Marshall⁷ investigated the distribution of myosin in skeletal muscle fibers. Although purified preparations of myosin were used to immunize the animals, the antisera were demonstrably impure by gel diffusion, containing one major and one or two minor components.

In the work by Marshall already cited,⁶ there are numerous technical improvements that make these papers valuable, not only as initial explorations in a new area of cytochemistry, but also as a source of methods for those who wish to use fluorescent antibodies for cytological purposes.

In the examples of the study of naturally occurring antigenic material described above, there has been a more or less complete escape from the circular immunological position. This has been made possible by the use of other than immunological methods, and also by the fact, emphasized by Hill and Cruickshank,^{2,3} that the localization of antigenic material to a given cell structure makes the unraveling of the antigenic composition of homogenates easier. It should be emphasized also that in each of these situations the antigenicity of the material investigated was species-specific. The localization of an enzyme or of the structural component of a cell must begin by the injection of suitable antigenic material derived from the species it is desired to study.

The most carefully studied antigen distributed in several species is that originally described by Forssman in 1911. The cellular distribution of this antigenic material in the tissues of the guinea pig, the cat, the dog, the mouse, and the chicken was recently described by Tanaka and Leduc.⁸ Antiserum was prepared in rabbits by the injection of sheep red blood cells or of horse kidney, materials both known to contain this antigen. The antigen was found in the form of small droplets in the cytoplasm of endothelial cells lining the blood vessels and scattered in their adventitial connective tissues. The largest amounts were found in the collecting tubules of the guinea pig kidney,

although its close association with the vascular tree renders it detectable in all organs. The proofs of the histochemical specificity of the staining reactions found in the various species investigated were adapted to the heterogenetic character of the antigen. The labeled antisera used did not react with tissues known not to contain Forssman antigen, such as rabbit or rat kidney. Normal rabbit serum globulin similarly labeled failed to stain Forssman-containing tissues. The reaction with Forssman-containing organs was inhibited by prior exposure of the tissue sections to anti-Forssman antiserum and the degree of inhibition paralleled the sheep-cell hemolysin titer of the serum. Absorption of aliquots of the conjugate with guinea pig kidney, with sheep red cells, or with water-soluble hapten prepared from horse kidney abolished the staining reactions. Stainable material was removed from the tissue sections by short exposures to ethanol but, on the other hand, prior heating of the tissue sections to boiling did not interfere with the reaction. All these known properties of Forssman antigen therefore were present in the material stained by labeled antiserum.

A final example of the use of fluorescent antibody in the study of tissue components and the first example of the application of these methods to a study of malignant tissue are contained in a recent publication by Weiler⁹ entitled (in translation) "Changes of the Serological Specificity of Liver Cells of the Rat during Carcinogenesis Induced by *p*-Dimethylaminoazobenzol." In this careful and revealing study, antiserum was prepared against mitochondrial and microsomal fractions of normal rat liver. There were complete cross reactions between these two fractions, although antisera to the microsomal fractions were produced in higher titer. It was demonstrated that hepatomata resulting from feeding of dimethylaminoazobenzol failed to react in complement-fixation tests with antiserum against normal cytoplasmic fractions. When such antisera were labeled with fluorescein, purified by absorption with cytoplasmic fractions from rat kidney, and applied to normal rat liver, the cytoplasm of the hepatic cells was uniformly reactive. However, in agreement with the complement-fixation reactions, hepatomata failed to react with these conjugates, indicating an antigenic loss in the tumor cells. Of most importance for this conference, it was found that after intervals shorter than the period necessary for the development of hepatomata, small groups of cells appeared in the liver that failed to react with normal anticytoplasmic conjugate, although by usual histological methods no change was apparent. This appears to be a clear demonstration of a change in the antigenicity of cells before overt malignancy is detectable.

To my knowledge, this is the first instance of a clear cytological demonstration of an antigenic difference between normal and malignant cells. It does not establish the existence of a different antigen in tumor cells, but it implies a difference in their antigenicity since, as they have lost a normal antigen, they have probably gained an abnormal one.

I have tried in this brief summary of the available examples to indicate both the possibilities and the difficulties inherent in attempting to use immunohistochemical methods as an approach to the investigation of malignant tissue. Further details and bibliography have been given in a review.¹⁰

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DIFFUSION-CHAMBER TECHNIQUES FOR STUDIES OF CELLULAR IMMUNITY

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The diffusion-chamber technique is a form of *in vivo* tissue culture in which cells may be grown within a porous chamber, nourished by body fluids, but separated by filters from direct contact with cells and tissues of the host.¹⁻³ The physical separation of graft from the host by filters results in a simplification of the complex *in vivo* environment of transplanted tissues and thus permits a more precise experimental analysis of certain problems of host-graft interaction.

Filters of cellulose derivatives are available from several sources.* The Millipore filters used in the studies to be discussed here are described as highly porous cellulose ester structures, containing numerous uniform and submicroscopic channels, the pore size of which can be controlled in the manufacturing process.⁴

It was found that filters of $0.45\ \mu$ average porosity or less allowed few if any host cells to pass through, whereas filters of $0.8\ \mu$ or greater average porosity permitted the free passage of host leukocytes and macrophages.

In one design of chamber² the filters are sealed to two sizes of plexiglas rings that fit together. They are sealed with a solution of plexiglas in acetone and sterilized in ethylene oxide. The implant or suspension is placed on the larger of the two filters, and the two rings with their attached filters are fitted together and sealed with the plexiglas-acetone cement. The chambers are placed subcutaneously or in the peritoneal cavity of mice and removed for fixation or biological assessment of cell survival after varying intervals of time. Duthie⁵ has used a simplified chamber for subcutaneous or intramuscular implantation in which the filters are attached to transparent adhesive tape. Another form of the diffusion chamber was designed to permit the introduction of suspensions of cells or the removal of cell-free filtrates for serological or chemical analysis.⁶ For the observation of living cells, the filters have been adapted for use with a transparent chamber inserted under the skin of mice.²

Our studies on homografts[†] within diffusion chambers grew out of an observation of R. M. Merwin in our laboratory that small unvascularized homografts of normal tissues survived in the subcutaneous site of mice because they did not initiate immunity.⁷ Such unvascularized homografts were soon destroyed, however, if the host was made immune by another graft which became vascularized.

Since nonvascularized homografts survive and do not initiate immunity in

* Available from the Millipore Filter Corp., Watertown, Mass., Carl Schleicher and Schuell Co., Keene, N. H., and The Wright Fleming Institute of Microbiology, St. Mary's Hospital Medical School, Paddington, London, England.

† Homografts are defined as grafts between animals of the same species but of different genotypes; isografts are grafts between animals of the same genotype.

the host it was not surprising to find that homologous tissues in diffusion chambers survived and did not initiate immunity. Intrachamber DBA lymphoma homografts of adrenal cortex in strain C57BL mice, for example, failed to initiate immunity in the host as indicated by lack of capacity to destroy unvascularized DBA Harderian gland tissue.⁸ Furthermore, even tumor tissue growing rapidly within diffusion chambers did not immunize the host.³ The exact mechanism of induction of immunity to homografts is unknown. Of possible bearing on our results is the work of Barrett and Hansen⁹ in which antigenic activity of the red cell seems to be associated with a large fragment of the red cell membrane.

In contrast to Merwin's results in which nonvascularized homografts were destroyed if the host was made immune, homografts in cell-impenetrable diffusion chambers survived not only in normal mice, but also in immunized animals, indicating that the filters blocked the destruction of the foreign cells by an immune host.^{2, 10} In these experiments mice were immunized by a single inoculation of a fragment of spleen approximately two weeks before implantation of the diffusion chambers. Homografts of Harderian gland, mouse embryonic lung, skin epithelium, or mammary adenocarcinoma, which I shall refer to as "target" tissues, were placed within the diffusion chambers, and these in turn were placed within the peritoneal cavity, or observed *in vivo* in transparent chambers. Observations *in vivo* were made of two grafts of Harderian gland in the same immunized animal, one within the diffusion chamber and the other in contact with tissues. The implants in contact with host cells were destroyed within 3 to 6 days, while grafts separated from host cells by a filter survived for the duration of the experiments (11 to 21 days).²

These results were confirmed and extended by experiments in which target cells were placed intraperitoneally in diffusion chambers and studied for survival either histologically or by biological tests.¹⁰

In another group of experiments target cells were combined with washed spleen cells in diffusion chambers. The chambers were placed in mice that were isologous with the target cells. There was extensive destruction of the target cells only if the spleen cells came from immunized mice. The experiments with spleen and target tissues, however, do not distinguish between cell-bound antibody or antibody manufactured and released into the serum.

By selecting filters of 0.8μ average porosity which allowed host cells to enter, it was possible to study the interaction of target cells in the chambers with leukocytes and macrophages from immune and nonimmune hosts. In these experiments destruction of the target cells was seen only if the cells entering the chambers came from immune hosts.¹⁰

It was concluded that under these experimental conditions antibodies cytotoxic to homografts were associated with cells. Not all of the types of cells that entered the chambers from the host appeared to participate in the destruction of the grafts. Destruction was not accomplished by phagocytosis. Histologic evidence suggested that the cells that destroyed homografts were lymphocytes. These results are in agreement with the observations of Mitchison¹¹ that cells from the lymphoid tissue of immunized mice possess the power of transferring homograft immunity.

In relation to virus studies, Bugher¹² has pointed out that the actual porosity of such membranes is uncertain, due both to the character of the structures and to the possible interaction of the material being filtered with the substance of the membrane.

The possible movement of some cells through the membranes indicated the desirability of considering the technical problems of construction of the chambers and of variation in pore size of the membranes used. Possible defects in the system of sealing the filters to the plastic rings must be considered, and alternative procedures are being studied. Do certain very motile cells escape through occasional pores which are considerably larger than the average size? Leukocytes and macrophages are found within the filter of $0.8\ \mu$ average porosity, but not within filters of lower porosity. A few leukocytes and connective tissue cells were found also to have entered chambers of $0.45\ \mu$ porosity which were empty when introduced into the host. Using double filters on each side of the implant, histologic evidence was obtained of the passage of leukemic cells.⁸

It was essential to discover whether filters that blocked the destruction of the homografts by immune hosts also would prevent the passage of antibodies. An indirect approach was made to this problem using animals that had been immunized by heterografts.

It is well known that tissue culture methods may be used to demonstrate the presence of humoral cytotoxic substances in the body fluids of animals that have been immunized by heterografts.¹³ Similarly, we have found that cytotoxins to heterografts may be demonstrated in the living animal through the use of diffusion chambers.¹⁰ In one series of experiments, mice were immunized two weeks earlier by a single subcutaneous injection of human cells of the HeLa strain. There was an accelerated destruction of HeLa cells, as compared with the nonimmune control animals.¹⁴

It seems reasonable to assume that the accelerated destruction of HeLa cells in the previously immunized mice was caused by cytotoxic antibodies to these heterografts. One would expect from these results that antibodies to homografts, if present, would also be capable of passing through the filters.

It seemed possible that the pores, even though initially permeable to humoral cytotoxins, might become blocked after a short time in the peritoneal cavity of the mouse, possibly by adsorption of protein material to the walls of the passages. Therefore, diffusion chambers containing HeLa cells that had been in nonimmune mice for 5 days were tested to see if the pores had become blocked. These chambers were transferred to previously immunized mice to see if the heteroantibodies would enter and bring about the destruction of the HeLa cells. It was found that the HeLa cells in the immune mice were severely damaged as compared to those in the nonimmune control animals.⁹ These results indicate that cytotoxic antibodies can pass through the pores of chambers that have been in the peritoneal cavity for at least 5 days.

In contrast to our failure to demonstrate cytotoxic antibodies to homografts in the body fluids of immunized mice, Gorer,¹⁵ with leukemic cells, and Billingham and Sparrow,¹⁶ with skin epithelium, have shown that serum antibody from hyperimmune mice can combine with these cells *in vitro* and inhibit their

growth on subsequent transfer into susceptible hosts. Gorer and Amos¹⁷ have demonstrated passive immunity in mice against C57BL leukemia E.L.4 by means of isoimmune serum.

Our inability to find cytotoxic antibodies to homografts may reflect only differences in the degree of immunity, in the choice of tissue, and in the dosage. Our animals, in contrast to theirs, were not hyperimmunized, yet the immunity was strong enough in our experiments to injure severely or destroy any graft to which host cells could gain access. We are cooperating with Bernard Amos, of the Roswell Park Memorial Institute, Buffalo, N. Y., in an attempt to determine whether diffusion-chamber methods can reveal cytotoxic antibodies in the body fluids of mice hyperimmunized to lymphoma cells.

Heterografts represent an extreme degree of tissue incompatibility. Humoral cytotoxic antibodies to heterografts may be detected in tissue culture or by diffusion-chamber methods as described above. Toolan¹⁸ has shown that cortisone may be used to overcome resistance to the growth of human tissues in heterologous hosts. We have been interested recently in whether diffusion-chamber methods could be used to block the induction of immunity by cells of the HeLa strain of human cervical carcinoma or by other heterografts.

In these experiments it was found that suspensions of HeLa carcinoma cells proliferated rapidly for 10 days in cell-impenetrable chambers but, unlike homografts, they showed areas of cellular degeneration at 15 days.¹⁴ The fact that many healthy cells could be found, even after 35 days, indicated that whatever was responsible for the cellular degeneration by 15 days was not usually sufficient to destroy the implants completely.

Experiments were next done to ascertain whether the mice had been immunized as a result of implantation of HeLa cells in diffusion chambers. If this were so, one would expect to find cytotoxic effects on cells in a second cell-impenetrable chamber implanted after removal of the first. In these experiments no greater destruction occurred in the animals that had previously carried heterologous cells than in the controls.

However, in view of the cellular degeneration of HeLa cells occurring after 10 to 15 days in diffusion chambers in normal mice, immunity was suspected. If immunity was weak, it seemed possible that, as postulated for homografts, cytotoxins would be found associated with cells rather than in the serum.

Further experiments were done to test this possibility. Cell-impenetrable chambers containing HeLa cells were removed from normal mice after 13 days, and second HeLa chambers using cell-penetrable filters (Type AA) were put into the same mice. These test chambers were removed after 7 days and compared with control HeLa chambers (Type AA) placed for 7 days in mice that had had a previous chamber without cells. The HeLa cells in the second chambers, using cell-penetrable filters, showed extensive cell destruction as compared with the controls.⁹ It appeared from these experiments that cell-bound antibodies are a more sensitive indicator of immune states than antibodies in the serum.

The weak immunity which developed against HeLa cells in diffusion chambers could have resulted from the escape of living HeLa cells or from the

passage of nonviable cellular components. If all cells were prevented from escaping, would the host nevertheless become immunized by nonviable cellular components? Could one, through selection of filters of the proper porosity, prevent immunization of the host by heterografts? These questions are under study.

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DISCUSSION: PART II

Michael Heidelberger, *Chairman*

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MICHAEL HEIDELBERGER: About 1920 an investigator named Gye caused considerable disquiet among the workers in the field of cancer by venturing to claim that antibodies could be formed to tumors. The complexities of the problem were not recognized at that time, and the evidence against Gye's findings seemed so overwhelming that nothing more was heard of it for many years. Finally, reports began to appear in the literature that immunological problems were, indeed, connected with cancer, and this monograph is one of the results of an attempt to inject some clarity into a very confused and difficult field.

The papers in this section of this publication deal with the various techniques of immunology and immunochemistry that may be applied and, to some extent, have been applied to the problem.

PIERRE GRABAR (*Pasteur Institute, Paris, France*): We have not mentioned one important consideration; namely, the differences among laboratory animals. If one takes ten animals and injects each of them with the same mixture of antigens in exactly the same way, perhaps two of the animals will react in the same manner. This is the principal pitfall in assessing all of the reactions that have been described so far.

Another consideration that should be mentioned is the fact that, with the exception of the complement-fixation reaction, we must use soluble antigens. In the precipitin reaction, and in the methods that I have described, only antigens in solution have been used, and no one knows whether, in cancer, some important antigens may be insoluble.

MICHAEL HEIDELBERGER: I was very much impressed by Maurice Rapport's results. I wonder, however, if the lung tissue or the extract contained a very small percentage of the same antigen that is present in your lymphosarcoma extract, whether you might not get the same kind of results. Even though you would establish a difference between most of the constituents present in the lung tissue, there might still be an identity of one or more constituents. You might obtain a qualitative difference. That in itself would be interesting, of course, but can you exclude that possibility?

MAURICE RAPPORT (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): No, my results do not exclude this possibility; the studies that I presented serve only as models. There is little doubt that lung and lymphosarcoma have certain antigens in common. However, the fact that the antibodies become progressively more discriminating after partial absorption does suggest that there are qualitative differences between them. The principal point I wished to emphasize is that, if a native antiserum fails to reveal such differences, whether quantitative or qualitative, such failure does not thereby guarantee the absence of any difference between two antigenic materials. By graded absorption, the very same antiserum can be made to disclose differences that are not discernible before such treatment.

Part III. Results of Immunological Techniques

THYROID-SPECIFIC AUTOANTIBODIES*

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In 1927, Hektoen and Schulhof demonstrated the serologic specificity of thyroglobulin that still serves as a model in the search for tissue-specific proteins. We previously reported that even crude thyroid extracts of different species, when carefully prepared and injected into rabbits, will elicit antibodies of considerable specificity for the thyroid extract of the species used for immunization,¹ similar to the thyroglobulin antisera first reported by Hektoen, Fox, and Schulhof,² as well as by Stokinger and Heidelberger.³ The reason for this tissue specificity of crude thyroid extracts might very well be their high content of thyroglobulin, sometimes attaining 80 per cent of the total protein.⁴ Recently, thyroid extracts were reported to be antigenic for animals of the homologous species.⁵⁻⁶ For instance, rabbit thyroid is antigenic for rabbits, dog thyroid for dogs, and guinea pig thyroid for guinea pigs.⁷ We believe these thyroid antibodies to be true autoantibodies mainly for two reasons: first, not only do pooled rabbit thyroid extracts elicit thyroid antibodies in normal rabbits, but thyroidectomized rabbits also develop circulating thyroid antibodies when injected with extracts of their own thyroid glands; and, second, rabbit thyroid antibodies produced in normal rabbits create marked changes in the thyroid glands of the animals treated. These changes could be demonstrated both serologically, by measurement of the decrease in the content of the thyroid-specific antigen in the thyroid glands, and by histological examination. Therefore, the antigenic constituents of the thyroid extracts used for immunization are either identical with those occurring in the intact thyroid gland or the changes are so minimal that the antibody is unable to distinguish between the antigen present in the extracts used for injection and the antigen as it occurs naturally in the thyroid gland.

The procedure of immunization was carried out in the following manner: thyroid glands were removed from the exsanguinated animals and either used immediately or frozen and stored at -30°C . After trimming away any excess connective or vascular tissues the glands were cut into fine slices with scalpels and ground in a Potter-Elvehjem tissue homogenizer, using a Teflon pestle. In general, 1 ml. of cold saline solution buffered with phosphate salts to pH 7.2 was added for each gram (wet weight) of tissue. The extract was lightly centrifuged to remove the larger particles. We prepared an emulsion consisting of equal parts of the saline thyroid extract and a mineral oil-Arlacel† mixture including acid-fast bacilli (Freund adjuvant) for immunization. Each of the

* The work reported in this paper was supported in part by Research Grant C-2357 from the National Cancer Institute, Public Health Service, Bethesda, Md.

† The Arlacel referred to is specially treated Arlacel "A" (mannite mono-oleate), Preparation No. 1B, manufactured by the Atlas Powder Company, Wilmington, Del.

four foot pads of the animal was injected with 0.05 ml. of this emulsion. In some instances a second or even third injection was given at monthly intervals, using in these cases 0.4 ml. of antigen emulsion, prepared in the usual manner, injected in 4 sites (0.1 ml. per site) intradermally into the back. The animals were bled approximately 3 to 4 weeks after each injection, and their sera were tested for the presence of thyroid antibodies.

In the search for the presence of circulating antibodies in these experimental animals, precipitation, complement fixation, and the tanned-cell hemagglutination test of Boyden⁸ were employed. The hemagglutination test, however, proved to be the most sensitive in our hands, and it allowed the detection of circulating thyroid antibodies in about 90 per cent of the rabbits, in contrast to precipitation and complement-fixation tests, which were positive in only about one third of the animals treated. These antibodies proved to be specific for extracts of the thyroid gland of rabbits and did not react with extracts of other rabbit organs or rabbit serum. This type of antibody directed against the rabbit's own thyroid gland is therefore characterized by an extreme degree of specificity that is demonstrable without additional adsorption or purification of the antiserum. Of course, one must be constantly aware of the possibility of the formation of isoantibodies, although we have not encountered any in our experiments so far. Even the possible occurrence of these group-specific antibodies can be avoided by injecting thyroidectomized rabbits with the animal's own thyroid extract as the immunizing agent.

As previously mentioned, thyroid antisera produced in the rabbit by injecting crude thyroid extracts of foreign species led to the formation of predominantly thyroid-specific antibodies. These thyroid antibodies exhibited cross reactions with the thyroid extract of various species in a somewhat unpredictable and irregular pattern similar to that shown in antisera directed against the purified thyroglobulin.⁹ The question arose whether the highly specific thyroid antibodies produced in the rabbit by means of rabbit thyroid extracts would show a similar tendency to produce cross reactions with thyroid constituents of other species. Thyroid extracts of various animals were prepared and tested by means of the tanned-cell hemagglutination test in the following manner:

Human cells of Group O, after being washed thoroughly 3 times with saline solution, were mixed as a 4 per cent suspension with an equal volume of a 1:25,000 dilution of tannic acid and kept at room temperature for 30 minutes. After that time the cells were washed again 3 times in phosphate-buffered saline, pH 7.2, and resuspended as a 2 per cent suspension. Equal volumes of diluted thyroid extracts previously kept for 2 minutes in a bath of boiling water were added to these tanned cells, and the mixture was kept at room temperature for another 30 minutes. Thyroid dilutions between 1:40 and 1:200 were used for the coating, depending upon the optimal concentration as determined in preliminary titrations. The coated cells were washed again 3 times in buffered saline solution containing 1 per cent of normal rabbit serum and adjusted to a final 1 per cent suspension. The antiserum dilutions were prepared in buffered saline solution containing 1 per cent normal rabbit serum. Equal volumes of antiserum dilution and the coated cells were mixed thoroughly and kept at

TABLE 1

HEMAGGLUTINATION TEST: TITRATION OF ANTI-RABBIT-THYROID RABBIT SERUM
No. 558 WITH CRUDE THYROID EXTRACTS OF VARIOUS SPECIES

Dilutions of rabbit serum No. 558	Rabbit thyroid extract	Human thyroid extract	Hog thyroid extract	Dog thyroid extract	Human serum
1:5	++++	++++	++++	++++	—
1:10	++++	++++	++++	++++	—
1:20	++++	++++	++++	++	—
1:40	++	++++	++++	++	—
1:80	++	++	++++	+	—
1:160	++	++	++	+	—
1:320	++	++	++	—	—
1:640	++	+	+	—	—
1:1280	++	—	+	—	—
1:2560	++	—	+	—	—
1:5120	—	—	—	—	—
1:10,240	—	—	—	—	—

room temperature for 2 hours and then overnight at 4° C. Several recordings of the resulting sedimentation pattern were made during the observation period.

As seen in TABLE 1, anti-rabbit-thyroid rabbit serum No. 558 reacts best with its homologous antigen, the rabbit-thyroid extract. However, cross reactions of various degrees of intensity occur when this antiserum is tested against thyroid extracts of other species. On the other hand, no cross reaction is observed with cells coated with human serum instead of human thyroid extract. In addition to human serum, cells coated with rabbit serum, hog serum, or dog serum were found to be negative and are omitted from the table. Therefore, even this highly specific rabbit anti-rabbit-thyroid antibody reacts with extracts of the corresponding tissue in other species, exhibiting classic organ specificity.

In addition to rabbits, dogs and guinea pigs were immunized with extracts of the thyroid gland of their own species. Circulating antibodies were produced in both dogs and guinea pigs, but not as consistently as in the rabbit. However, the histological changes of the thyroid gland of the immunized animals were similar to those of the rabbit (FIGURES 1, 2, and 3).

As soon as the histological changes in the animals immunized with thyroid extract of their own species became apparent, the similarity of experimentally produced thyroiditis in animals to chronic thyroiditis as it occurs in humans was recognized. Therefore, serum specimens of patients suffering from chronic thyroiditis and other thyroid pathology were collected and examined for the possible presence of antibodies against the human thyroid. Sera of four patients* with thyroiditis indeed contained thyroid antibodies when tested in exactly the same manner as that described for the sera of the experimental animals. The sera of about 20 patients with other types of thyroid disease and about 150 other controls were negative. The exact circumstances that allow the demonstration of these human thyroid antibodies in patients suffer-

* Since this paper was presented on January 5, 1957, thyroid antibodies have been found in the sera of about 40 additional patients with thyroiditis. A report of this aspect of our studies is in press.¹⁰

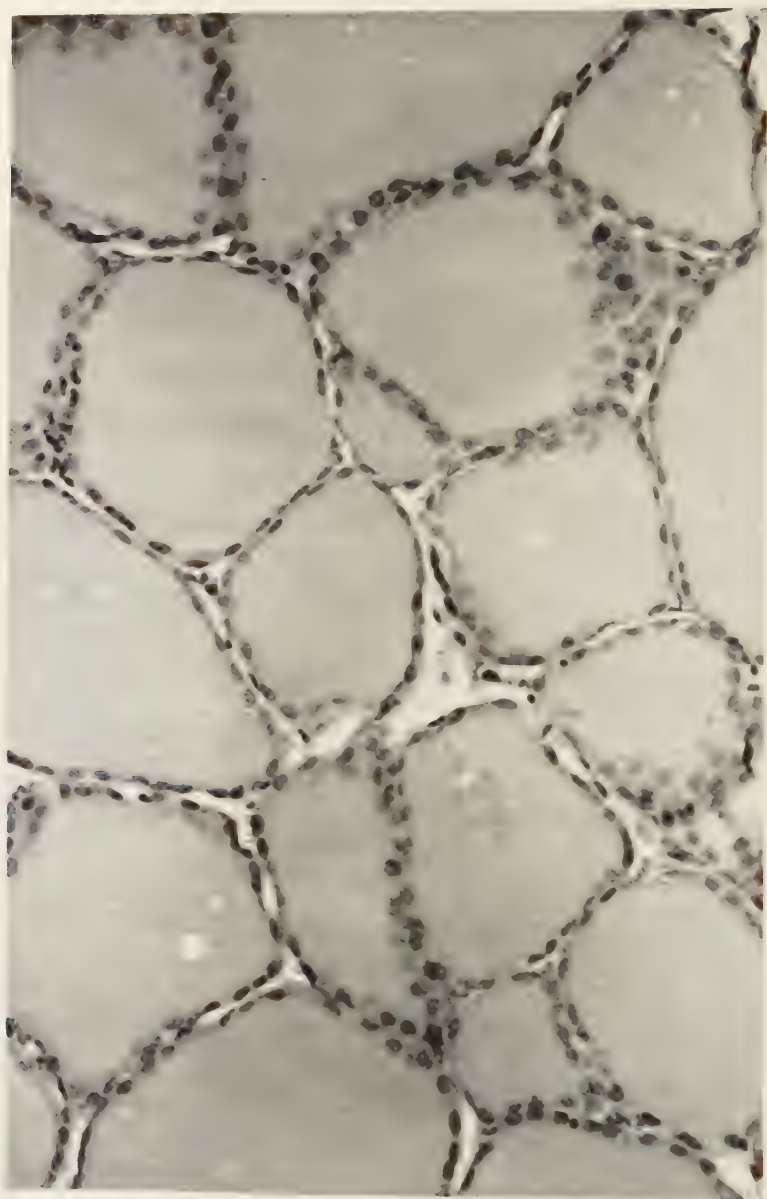


FIGURE 1. Section of the thyroid gland of a normal rabbit. $\times 190$.

ing from chronic thyroiditis and especially their disappearance following medical or surgical treatment must be studied further. However, there can be no question that circulating antibodies are demonstrable in the serum of some patients with chronic thyroiditis. The tanned-cell hemag-

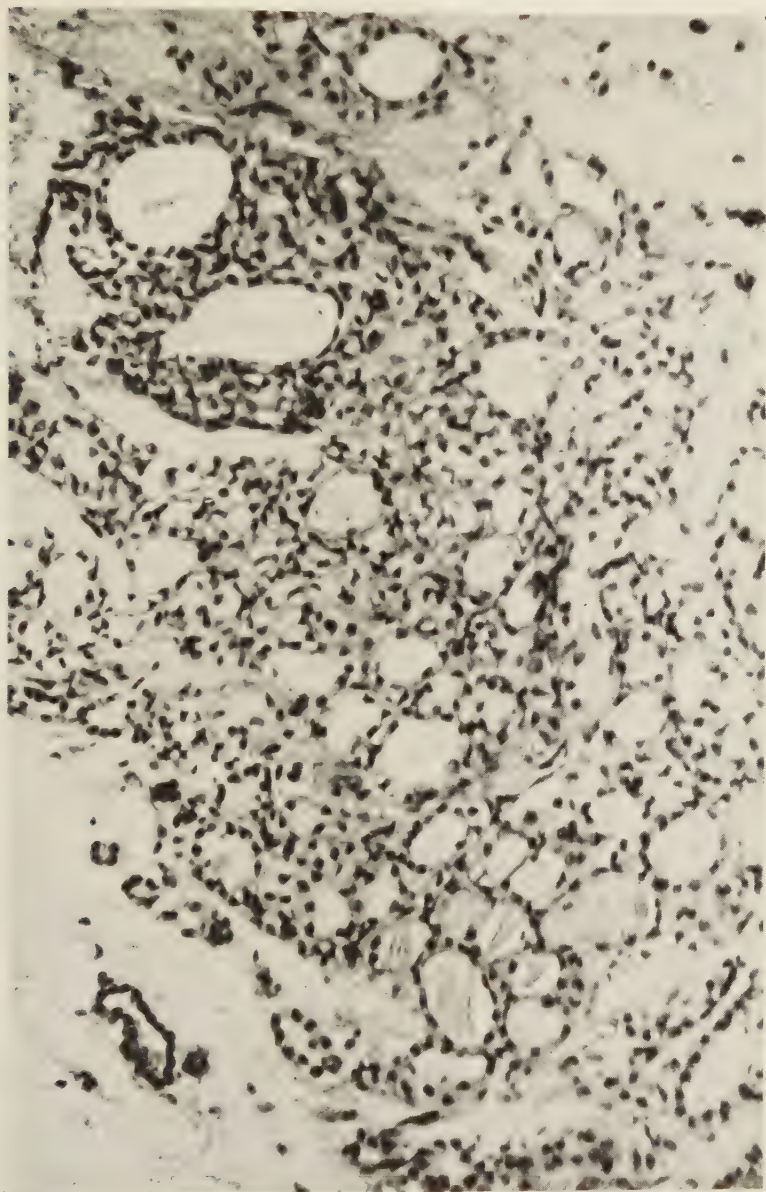


FIGURE 2. Section of the thyroid gland of rabbit No. 297, which had been treated as follows: December 28, 1954—injected intradermally with 0.2 ml. of pooled rabbit-thyroid crude extract with Freund adjuvant; February 22, 1955—injected intradermally with 0.4 ml. of pooled rabbit-thyroid extract with Freund adjuvant; and March 1, 1955—thyroid glands removed. The section shows the infiltration of eosinophilic and lymphoid cells. $\times 190$.

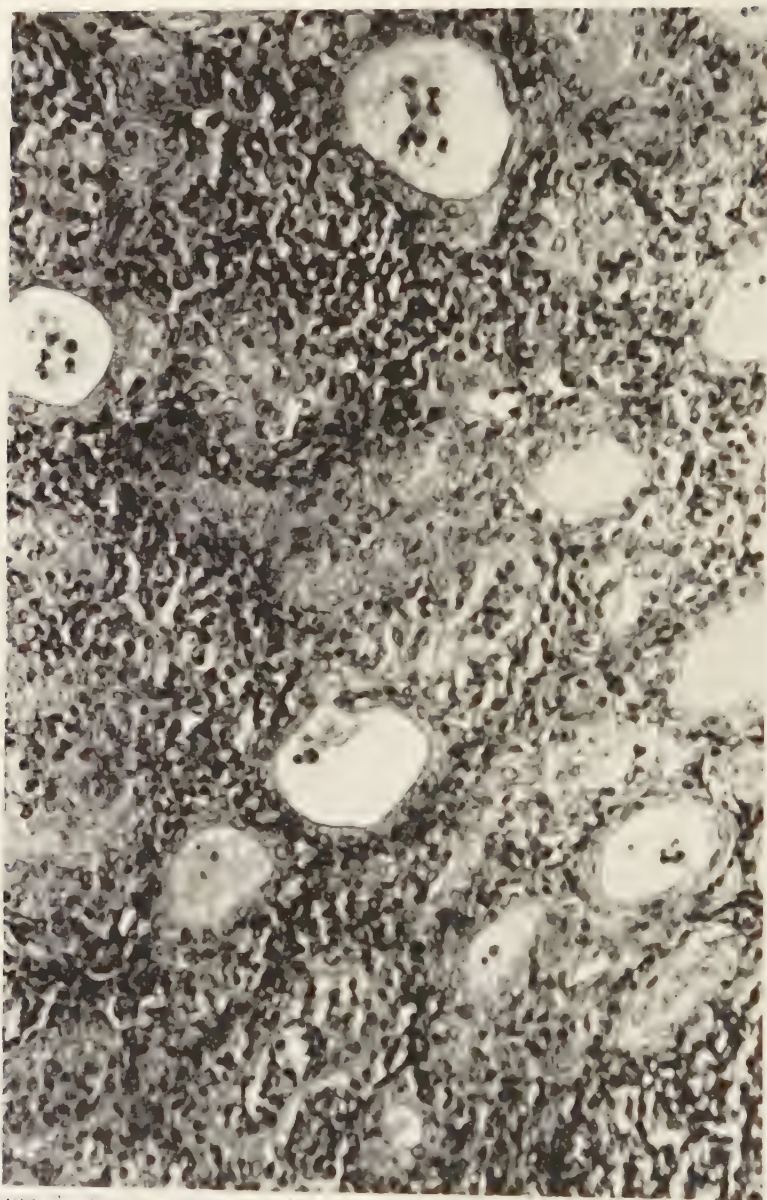


FIGURE 3. Section of the thyroid gland of dog No. 1196, which had been treated as follows: October 27, 1954—injected intradermally with 0.4 ml. of dog thyroid extract with Freund adjuvant; December 24, 1954—injected intradermally with 0.4 ml. of dog thyroid extract with Freund adjuvant, but omitting acid fast bacilli; January 20, 1955—injected intradermally with 0.4 ml. of dog thyroid extract with complete Freund adjuvant; and May 23, 1955—thyroid glands removed. This section shows marked inflammatory changes. $\times 190$.

glutination test again proved the most sensitive tool for the demonstration of thyroid autoantibodies, paralleling our experience with animal sera. Only 1 of the 4 sera gave precipitation and a slight degree of complement fixation. This phase of our investigation is being published elsewhere.¹¹ Recently, Roitt, Doniach, Campbell, and Hudson¹² reported the presence of thyroid antibodies in the sera of patients suffering from the so-called Hashimoto type of thyroiditis. These authors observed precipitation by mixing human thyroid extracts with the serum of thyroiditis patients. They did not obtain precipitation with the serum of patients suffering from other thyroid diseases. They feel that the Hashimoto type of thyroiditis results from an antigen-antibody reaction, an opinion that is supported further by the fact that these patients have a high gamma-globulin content in their sera.

In view of the fact that rabbit thyroid autoantibodies gave considerable cross reaction with thyroid extracts of various animals, we wondered whether the human thyroid autoantibodies also would show cross reaction with thyroid extracts of other species. The serum of a patient (Tho) containing potent thyroid antibodies was tested with thyroid extracts of various species by means of the tanned-cell hemagglutination technique in exactly the same manner as was the serum of animals with experimental thyroiditis. The results of such a test are shown in TABLE 2.

As can be seen from the data in this table, the serum containing antibodies of high titer against human thyroid exhibits some cross reaction with the thyroid extracts of various species, though the homologous antigen, in this case human thyroid extract, is by far the strongest. Again the cross reaction is thyroid specific, since no agglutination is observed with cells coated with hog serum. In addition, human serum, rabbit serum, and dog serum were negative and are omitted from the table. Consequently, in the case of thyroiditis we have an example of a disease that can be produced in experimental animals by artificial autoimmunization. Thyroid antibodies against human thyroid extracts are found in the human disease of chronic thyroiditis. It is therefore suggestive

TABLE 2
HEMAGGLUTINATION TEST: TITRATION OF HUMAN THYROIDITIS SERUM (Tho)
WITH CRUDE THYROID EXTRACTS OF VARIOUS SPECIES

Dilutions of human serum (Tho)	Human thyroid extract	Rabbit thyroid extract	Hog thyroid extract	Dog thyroid extract	Hog serum
1:5	+++	++	++	+	—
1:10	+++	+	++	+	—
1:20	+++	—	+	—	—
1:40	+++	—	—	—	—
1:80	+++	—	—	—	—
1:160	++	—	—	—	—
1:320	+	—	—	—	—
1:640	+	—	—	—	—
1:1280	+	—	—	—	—
1:2560	—	—	—	—	—
1:5120	—	—	—	—	—
1:10,240	—	—	—	—	—

that the pathogenesis of human thyroiditis might be connected with the appearance of antibodies against constituents of the human thyroid gland itself. Only further large-scale investigations may disclose whether the knowledge gained by the successful autoimmunization against thyroid tissue might be applied in attempts to elicit autoantibody formation against constituents of other tissues, such as malignancies.

For many years our laboratory has been interested in the serologic analysis of constituents of malignant tissues, among them cancer of the thyroid gland. This study was undertaken by injecting rabbits with saline extracts of cancerous human thyroid tissue obtained either at surgery or post mortem. The antisera obtained in this way were compared with antisera produced in rabbits by injecting normal human thyroid extract. The electrophoretic analysis of extracts of the normal thyroid gland differs impressively from that of thyroid cancer extracts.⁷ However, it is surprisingly difficult serologically to pinpoint constituents that are specific only for cancer of the thyroid. Antisera produced by immunization of animals with thyroid extracts of a foreign species contain, in most instances at least, a wide spectrum of antibodies against many different constituents of the organ extract. The serum of thyroiditis patients containing thyroid autoantibodies of great specificity offers a new tool to study the relationship of antigens as they occur in normal human thyroid glands on the one hand and in malignancies on the other. The advantage of using an autoantibody in contrast to a heteroantibody against human thyroid produced in rabbits is illustrated in the following experiment.

Rabbit serum No. 440, prepared by the injection of human thyroid extract into rabbits, appeared to be specific for thyroid extracts on the basis of complement-fixation and precipitation tests. This serum was compared with that obtained from a thyroiditis patient (Tre) in a tanned-cell hemagglutination test. Two antigens, human thyroid extract and normal human serum, were used. The end points of antiserum titration are given in TABLE 3.

Evidently the rabbit antiserum cross reacts to some extent with human serum when tested by the highly sensitive tanned-cell hemagglutination test, in contrast to the human thyroiditis serum, which does not. Therefore the use of a new, highly specific reagent, namely, human thyroid autoantibody might permit a better understanding and analysis of the antigenic constituents of normal and malignant thyroid extracts.

TABLE 3
HEMAGGLUTINATION TEST: SPECIFICITY OF ANTISERA
AGAINST THE HUMAN THYROID

	Thyroiditis patient (Tre)	Anti-human thyroid rabbit serum No. 440
Human thyroid extract	2560	5120
Human serum	0	320

Figures represent the greatest dilution of antiserum giving 1+ agglutination of tanned human Group O erythrocytes coated with the optimal concentrations of each antigen (human thyroid extract—1:100; human serum—1:200).

Acknowledgments

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ACQUIRED TOLERANCE IN NEWBORN MICE

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Acquired tolerance of skin homografts may be brought about in the mouse by inoculating fetuses with tissue-cell suspensions.¹ It has now been shown that equally satisfactory results can be obtained by the intravenous injection of such material into *newborn* mice.² This method is an improvement upon the old one, insofar as it takes advantage of a reliable route of injection and circumvents the problem of immediate postoperative mortality among the injected fetuses. The technique of injecting the orbital branch of the anterior facial vein with living homologous cells of the adult spleen has been described elsewhere.²⁻³

With A-strain litters as recipients and CBA mice as donors, a very high proportion of mice injected within 24 hours of birth fully tolerated their CBA skin grafts when these were transplanted to them 6 to 7 weeks later (TABLE 1). There was, however, a sharp decline in the proportion of tolerant animals among mice injected after the first day of life. The majority behaved as if they had never been injected at all; their grafts indicated that the hosts had developed neither immunity nor tolerance as a result of the postnatal injection of foreign cells. This "neutral period" evidently ends some time before the seventh day after birth, for mice injected with homologous spleen cells on that day were found to be weakly immune.

The sharp decline in the proportion of tolerant mice contrasts vividly with the finding that, in the rat, tolerance of skin homografts can be induced by subcutaneous injection as late as the fourteenth day after birth.⁴ This difference between the two species is emphasized further by the demonstration that, in the newborn mouse, the subcutaneous route of injection is wholly ineffective in inducing tolerance (TABLE 2). The postnatal "adaptive period" is here so short that the speed with which the antigenic stimulus is distributed assumes considerable importance (in the adult mouse, the intraperitoneal and subcutaneous routes of injection are quite as effective in *immunizing* the recipients as is the intravenous route⁵).

The fact that the subcutaneous implantation of tumor fragments into newborn mice will render a fairly high proportion of the recipients tolerant of the tumor is presumably due to the special properties of neoplastic tissues.¹ The relationship between the time of birth and the end of the tolerance-adaptive period is clearly not a rigid one, and may even vary from strain to strain. It must therefore be stressed that the data given in TABLE 1 need not apply to other donor-host combinations. For example, when the recipient strain was CBA and the donor strain was A, the percentage of tolerant mice was roughly one half of that obtained in the reverse combination, whereas the CBA \rightarrow AU combination yielded no highly tolerant mice at all. The possible role played by the degree of antigenic disparity between donor and host in the induction of tolerance already has been discussed.³

Of special interest is the finding that a number of Strain A mice made tolerant

TABLE 1
INDUCTION OF TOLERANCE IN NEWBORN A-STRAIN MICE WITH CBA
SPLEEN CELLS INJECTED INTRAVENOUSLY

Age at injection	Number of mice grafted	Per cent tolerant	Per cent highly tolerant*
12 hr.	39	95	80
24 hr.	14	100	78
48 hr.	16	56	31
4 days	15	13	7

* Highly tolerant mice are those whose grafts survived for at least 50 days after test operation.

The actual dosage of cells administered to each recipient ranged from 4 to 10 million.

TABLE 2
THE EFFICACY OF OTHER ROUTES OF INJECTION OF CBA SPLEEN CELLS
IN CONFERRING TOLERANCE ON NEWBORN A-STRAIN MICE

Route	Age at injection	Number of mice grafted	Per cent tolerant	Per cent highly tolerant
Intravenous	12 hr.	39	95	80
Subcutaneous	12 hr.	23	0	0
Intraperitoneal	12 hr.	29	65	24

by the injection of adult CBA spleen cells remained extremely backward and emaciated, and finally died within two to three months of birth. Such mice invariably displayed striking anatomical deficiencies in their lymphoid tissues; in fact, many of the normally prominent lymph nodes were completely absent. The hypothesis that this state of affairs may have been brought about by an immunological reaction of the spleen cells of the donor against the tissues of the host is supported by the demonstration that the donor cells do indeed settle down in the lymph nodes and spleens of mice rendered tolerant by intravenous injection.² Apparently healthy tolerant mice were found to suffer also from lymphoid abnormalities, though on a much reduced scale, whereas in incompletely tolerant animals such abnormalities were barely identifiable. In its extreme form the reaction has led to the death of whole litters two to three weeks after birth.

The possibility that a graft may react against its host (provided it is properly equipped to do so) was first suggested by workers studying the destruction of kidney homografts^{6,7} and was given reality by the transplantation of normal lymph node cells into tolerant mice.⁸ The present findings emphasize the practical importance of graft-versus-host reactions, and suggest a cautious approach to the problem of restoring immunological reactivity to hypogammaglobulinemic humans.¹⁰

Relevant to the above considerations are reports that many mice injected intravenously with homologous bone marrow immediately after whole-body X irradiation die after an initial period of well-being, during which the donor cells exercise a protective effect.^{11,12} Here, by a very different method,

the hosts have also been rendered incapable of reacting against the donor cells, which are themselves admirably equipped to respond to tissue antigens of the hosts. Some investigators have considered the possibility of a graft-versus-host reaction in order to explain this delayed mortality,^{11, 14} and our own observations on tolerant mice make such an interpretation highly plausible. While this work was in progress Simonsen¹⁵ has presented unequivocal evidence of graft-versus-host reactions in birds that are essentially similar to those observed by us following the inoculation of newborn mice with adult lymphoid tissue cells.

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THE DISTRIBUTION AND IMMUNOCHEMICAL PROPERTIES OF HUMAN TISSUE AND TUMOR ANTIGENS*

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Until recently, immunochemists have avoided studying the antigens of human tissues and tumors. Two main factors have been responsible for this reluctance: (1) tissue antigens are poor inducers of antibody, and (2) the large number of tissue antigens, none of which has as yet been purified, defies analysis by classic immunochemical techniques.

The development of new immunological methods has led us to re-evaluate the possibility of obtaining meaningful data in a field that has long been plagued by technical difficulties. Among these new methods are the immunization procedure developed by Freund¹ and the various gel-diffusion techniques.

We have found that frequent injection of rabbits with large amounts of tissues or tumors in Freund adjuvant yields potent precipitating antisera in a large percentage of the animals. The Ouchterlony gel-diffusion technique² has provided us with a tool that enables us to analyze these antisera even when several antigen-antibody systems react simultaneously. Furthermore, it permits the identification of similar antigens in different tissues.

Unfortunately, the papers dealing with this important technique are scattered throughout many journals, most of them European, and the only review easily accessible to American oncologists is outdated.³ Therefore, before I present the results obtained in our laboratory, it might not be amiss to describe our methods and how we interpret the data obtained by them.

The Ouchterlony Gel-Diffusion Technique

We use the Ouchterlony gel-diffusion technique² with minor modifications.⁴ Five gm. of Difco agar† are suspended in 600 ml. of saline buffered at pH 7 with phosphate buffer and are brought into solution in the autoclave. The hot solution is immediately clarified by centrifugation and redissolved. After adding merthiolate to a final concentration of 0.01 per cent, a layer of gel is prepared by pouring 9 ml. in a flat-bottomed Petri dish 9 cm. in diameter. After this layer has solidified, stainless steel penicillin assay cylinders are arranged at the corners of a square, with a fifth cylinder in the center. Another layer of agar (10 ml.) is poured on top of the first. The cylinders may be arranged in other patterns whenever necessary. In our work, the distance from the center of the antibody reservoir to the center of the antigen reservoir is always 1.8 cm. Two tenths ml. of antiserum or antigen are transferred to the cylinders by means of a pipette. After 1 to 3 days, while the cylinders still contain some solution, they are refilled with 0.2 ml. of antigen. The plates are kept at 25° C.

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† Product of the Difco Laboratories, Detroit, Mich.

Most good antisera produce at least 1 precipitin line after 1 or 2 days if the concentration of antigen is optimal. Antisera that produce no lines within 1 week are discarded. The addition of merthiolate to antigen and antibody solutions prevents the growth of bacteria and molds; no other precautions are necessary to maintain sterile conditions.

Interpretation of Data

When antigen and antibody diffuse toward each other through a gel, a specific precipitin line is formed in the region of optimal proportion; when several antigens and their specific antibodies diffuse toward each other, several lines will be formed. The number of lines is dependent on the number of antigens that are capable of reacting with antibody under the experimental conditions. Since antisera usually do not contain antibody against all or even most tissue antigens in a mixture, it follows that the number of precipitin lines represents a minimum, and that a single line cannot be used as a criterion for the purity of an antigen preparation, or for the possible number of antigens in this preparation.

The chances that 2 precipitin lines will be superimposed are small, since the position of a line depends on the concentration of antigen, its diffusion coefficient, and the optimal proportion of antigen and antibody for the system at equivalence. Nevertheless, 2 lines occasionally will appear as 1, but a change of antigen concentration will usually resolve them. This phenomenon is illustrated in FIGURE 1, in which an antileukemic cell serum produces 1 thick line

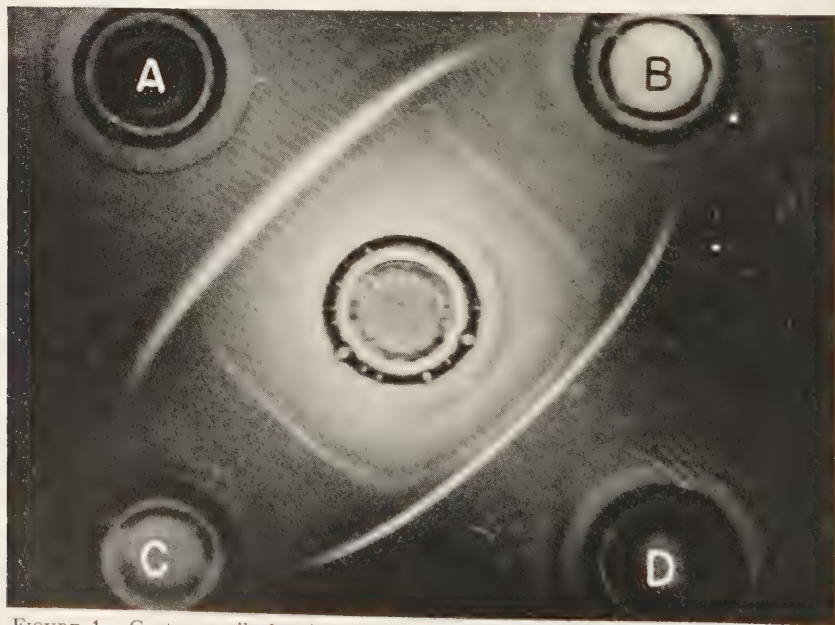


FIGURE 1. Center, antileukemic cell A antiserum absorbed with plasma: (A) leukemic cell A (1 mg./ml.); (B) leukemic cell B1 (1 mg./ml.); (C) leukemic cell B2 (1 mg./ml.); (D) leukemic cell A (2.5 mg./ml.).

with the homologous leukemic cell extract at 1 mg. ml. (A), but 2 lines at 2.5 mg./ml. (D).

Close scrutiny of a precipitin line can teach us much about the nature of the antigen-antibody system. Three features should be observed: (1) the position of the line with respect to the antigen reservoir; (2) the curvature of the line; and (3) the density of the line.

(1) *Position.* With a given antiserum, changes in the concentration of the antigen will influence the position of the resulting line; increasing the antigen concentrations will move the line closer to the antibody reservoir. Theoretically, it is possible to plot a curve that relates the position of the line to the concentration of antigen. Experience has shown, however, that in practice the quantitative aspect of the Ouchterlony technique is unsatisfactory. Nevertheless, marked differences in the position of a line obtained with samples of the same antigen and its antiserum are indicative of considerable differences in antigen concentrations. Better results can be obtained by determining the minimal antigen concentration that still produces a precipitate within a given time. For example, if one solution of antigen can be diluted tenfold before the line disappears and a second solution can be diluted fortyfold, it follows that the latter is approximately four times as concentrated.

(2) *Curvature.* On examining the curvature of precipitin lines formed by different antigens, we noticed that some were convex with respect to the antibody reservoir, some were concave, and others had little if any curvature. The curvature is independent of the concentration of antigen or antibody. These differences in curvature now have been correlated with the diffusion coefficient of the antigen and, since this coefficient is inversely proportional to the molecular weight, it is possible to estimate the size of an antigen molecule. From geometrical considerations it follows that a precipitin line must curve away from the antibody reservoir if antigen diffuses more slowly than does antibody, since the ratio of antigen to antibody remains constant along the locus of the line. The reverse is true when antigen diffuses more rapidly than does antibody, and the line will be straight if both reagents diffuse at the same rate. Evidence for this correlation has been obtained by a study of seven highly purified proteins with known diffusion coefficients and molecular weights, and a mathematical analysis has proved its generality.⁵

(3) *Density.* The density of the precipitin line, with the optimal concentration of antigen, is a relative index of the antibody content of the antiserum; the more potent the antiserum, the denser the resulting line. At times, the use of antigens in high dilution may result in a line that is close to the antibody reservoir. Two factors may be responsible: either the antiserum is very weak, or the antigen diffuses very rapidly. If the resulting line is faint, a weak antiserum is more probable; if the line is dense, considerable amounts of antibody must be present, and the position of the line is due to rapid diffusion of antigen. The curvature of the line should verify this.

Further information about an antigen may be obtained by specific staining of the precipitin lines. It has been shown that antigens rich in carbohydrate or lipid form precipitin lines that can be stained by specific dyes.^{6, 7} Recently, a protein stain has been described,⁸ but since the immune precipitate is composed

primarily of antibody protein, this stain has only esthetic value. One word of caution about the carbohydrate stains may be in order. Gamma-globulin and, presumably, antibody, contain carbohydrate;⁹ a positive stain of dense immune precipitates may result in erroneous interpretations if this factor is not considered.

The present discussion has been limited to the analysis of one antigen diffusing from one antigen reservoir. However, the main advantage of the double-gel diffusion technique as developed by Ouchterlony resides in its ability to permit the comparison of antigens or antigen mixtures in adjacent reservoirs. The resulting patterns, first described by Ouchterlony,² are: coalescence, partial coalescence, or intersection of the lines (FIGURE 2). If the same antigen is placed in adjacent reservoirs (C and D) and the homologous antiserum in the center reservoir, the lines will coalesce. Consequently, when we observe such a pattern, it may be concluded that the two antigens are either closely related or identical. If both reservoirs are filled with antigen at the same concentration, the axis of symmetry through the point of fusion will be equidistant from the two antigen reservoirs; if one reservoir (C) contains a more concentrated solution, the axis will be displaced toward the reservoir containing the more dilute solution. A line formed by an antigen is always deflected by the same antigen at higher concentration in the adjacent reservoir. Failure to grasp this basic principle may result in the publication of precipitin patterns that are

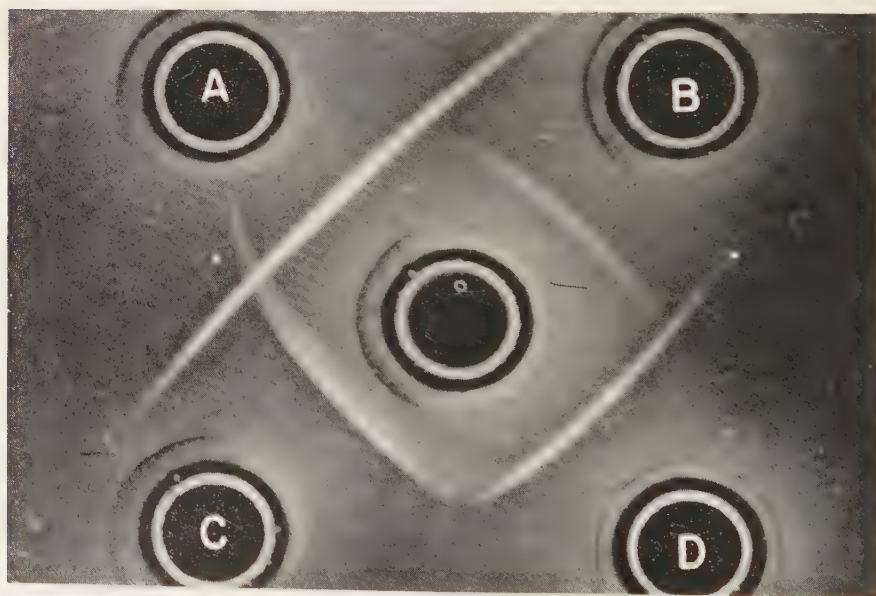


FIGURE 2. Center, antibodies against human serum albumin and γ -globulin: (A) γ -2 globulin; (B) Bence Jones protein; (C) serum albumin (0.1 mg./ml.); (D) serum albumin (0.02 mg./ml.).

The intersection of the lines formed by the Bence Jones protein and the serum albumin is not visible in this reproduction.

theoretically impossible (see, for example, FIGURE 1 of Seligmann¹⁰). If two adjacent reservoirs contain unrelated antigens (A and C in FIGURE 2), and the antiserum contains antibody against both of them, the resulting lines will intersect. If one reservoir (A) contains the immunizing antigen, and the adjacent reservoir (B) a cross-reacting protein, the two lines will coalesce partially and a spur will extend beyond the point of fusion. I have recently discussed the theoretical aspects of this phenomenon and how it can be used to study structural differences among related proteins.¹¹

It is possible to compare several antisera by placing them in the peripheral reservoirs, and the antigen or antigens in the center. If the antisera react with the same antigenic molecule the lines will coalesce, whether they react with the same or different antigenic groupings on that molecule (see figures 13 and 14 in Korngold and Lipari¹²). If the antisera are directed against unrelated molecules the resulting lines will intersect. Complications may result when the antigen is a mixture of immunologically related molecules.

The double-gel diffusion technique is almost foolproof when properly used. Several precautions, however, should be taken to prevent such artifacts as split lines or pseudospurs that confuse the patterns. Two of these precautions are: (1) the concentration of antigen should be such that the precipitin line will not move too close to either the antigen or the antibody reservoir, and (2) all reservoirs should be refilled while they still contain some liquid.

The Application of the Ouchterlony Technique to the Study of Neoplasia

The injection of rabbits with human tissues may result in the formation of antibodies against many tissue proteins (FIGURES 3 and 4) and also of plasma proteins (FIGURE 3). However, we are not interested in the antibodies against the latter, and we remove them by absorbing the antisera with lyophilized, pooled human plasma. (Originally we used the procedure of Bjørklund,¹³ in which the plasma is incorporated in the agar. However, our experience has confirmed the observation of others¹⁴ that not enough plasma can be introduced in the agar to remove all antibody.)

If, after absorption, the antiserum still produces precipitin lines with human plasma, the procedure is repeated until all such antibody has been removed (FIGURE 3). The remaining antibodies capable of reacting with tissue proteins vary from one antiserum to the next, even if the rabbits are immunized with the same preparation.

The antigenic composition of a tissue or a tumor is a reflection of its cellular heterogeneity as well as of the antigenic complexity of the individual cell. The number of antigens contributed to a tumor extract by stroma or normal tissues varies in different tumors and in human material. This is a factor that cannot be determined experimentally. Let us therefore examine human tumors grown in cortisone-treated rats as a model. The extracts of four such tumors (HS No. 1, HEp No. 3, H. Emb. Rh. No. 1, and a melanoma)¹⁵ were placed in the peripheral reservoirs, and an antiserum capable of reacting with two rat tissue antigens was placed in the central cup (FIGURE 5). From the resulting precipitin pattern it follows that one of the antigens is a relatively large molecule because of its convex curvature with respect to the antibody cup.⁵ This antigen

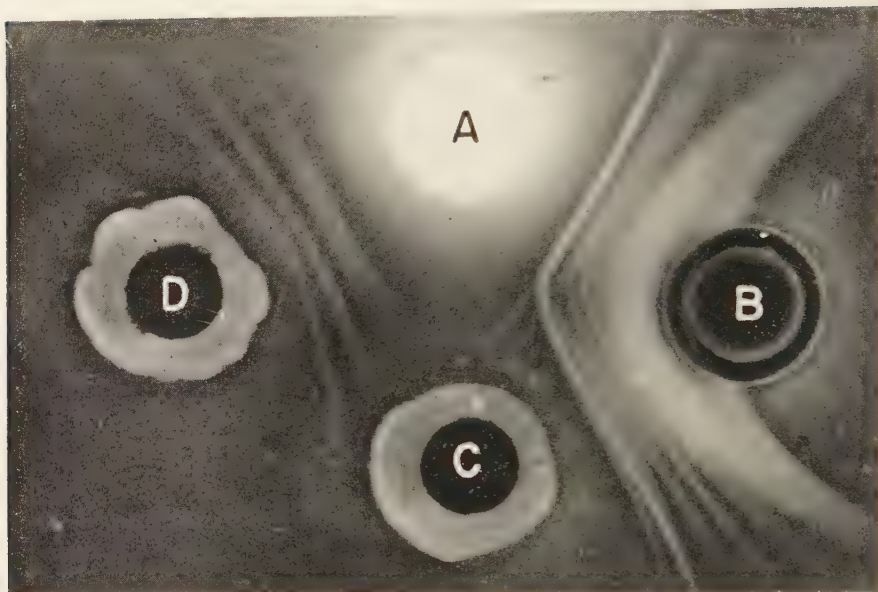


FIGURE 3. (A) Carcinoma of the breast; (B) anticarcinoma of the breast serum; (C) plasma; (D) anticarcinoma of the breast serum absorbed with plasma.

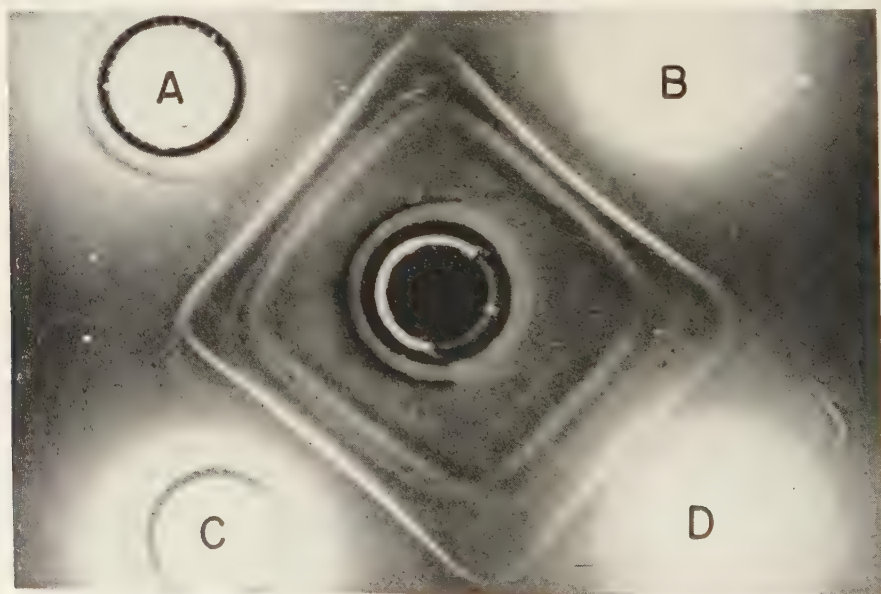


FIGURE 4. Center, antimelanoma serum absorbed with plasma: (A) melanoma (20 mg./ml.); (B) ovarian carcinoma No. 3 (40 mg./ml.); (C) reticulum cell sarcoma (40 mg./ml.); (D) uterine fibroma (40 mg./ml.).

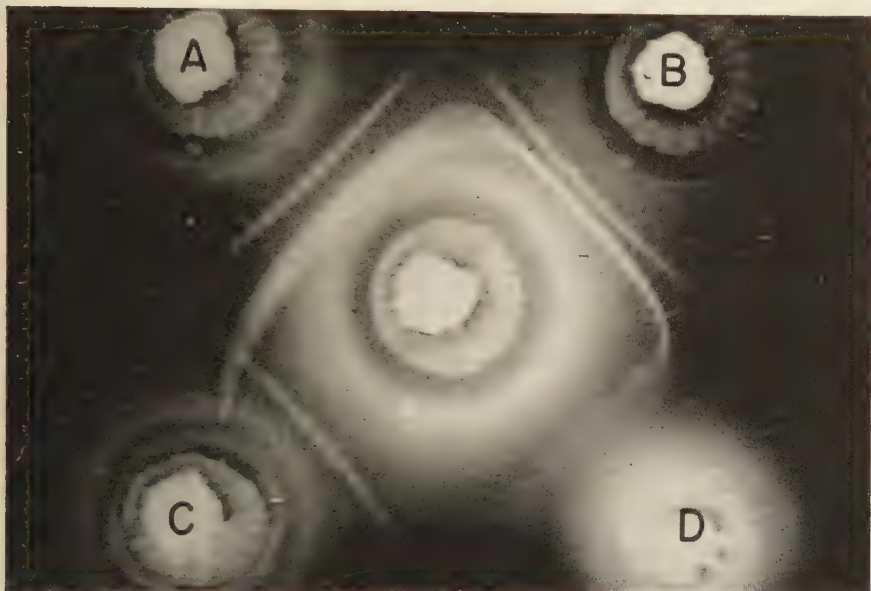


FIGURE 5. Center, anti-rat tissue serum absorbed with rat plasma: (A) HEp No. 3 (40 mg./ml.); (B) HS No. 1 (40 mg. ml.); (C) H. Emb. Rh. No. 1 (40 mg./ml.); (D) human melanoma grown in rats (40 mg. ml.).

is present in approximately the same concentration in three of the tumors and is not detectable in the melanoma. The second antigen is present in such a high concentration in HEp No. 3 that the precipitin line has moved to the rim of the antiserum reservoir. It is less concentrated in HS No. 1 and, in the melanoma, it is so dilute that the line is barely visible (note the split-line artifact). However, no line is formed with the extract of H. Emb. Rh. No. 1. This experiment clearly demonstrates that the contribution of antigen from surrounding tissues may vary in tumors grown at the same location; not only the concentration, but even the relative distribution of the antigens varies (when these human tumors are grown in eggs subsequent to their sojourn in rats, the rat antigens are lost immediately, indicating that they are not synthesized by the tumor cells).

It is important to ascertain whether an antigen is really derived from epidermal cells or from contaminating connective or muscular tissue. This can be done by comparing the tumor extract with the extracts of normal tissues that are rich in connective tissue or muscle. If these normal tissues have very little of a given antigen, and if the tumor contains it in large amounts, then this antigen was not derived from normal tissues.

The comparative study of tissue antigens requires a procedure that permits the antigens to be extracted reproducibly. The method we use¹⁵ appears to meet this requirement; aliquots of the same tissue, processed and extracted at different times, yield very similar data. This is illustrated in FIGURE 1. In

this example whole blood from a patient with leukemia was divided into 2 portions; the leukocytes were isolated, and their antigens were extracted. It may be seen that both extracts contain the same component at the same concentration. Furthermore, extracts of tissues of similar antigenic composition usually contain the same antigens in approximately equal concentration (FIGURE 4).

The claim that an antigen is absent from a tissue extract has little meaning unless we first define the quantitative limits of the technique. Our extracts are always dialyzed against distilled water, and the water-soluble proteins are lyophilized. The initial concentration of all extracts to be tested is 40 mg. ml.; by subsequent serial dilution we determine the end point at which an antigen no longer produces a line. For example, if a given antigen from a carcinoma of the ovary still can be detected with a concentration of 1 mg. ml., whereas it is not seen at 40 mg./ml. of another tissue extract, it can be said that at least 40 times more antigen is present in the tumor.

Our study of tissue and tumor antigens has been complicated by the finding that the concentration of some tissue antigens varies for the same organ of different persons. For example, the carcinomas of the cervix and the normal uterine tissues from patients X and Y were tested for their ability to react with an antiserum against ovarian carcinoma No. 3. This antiserum still reacted with as little as 0.1 mg. ml. of the homologous tumor. From FIGURE 6 it can be seen that at 40 mg. ml. the carcinoma of the cervix X and uterus X produces a strong line; the same tissues from patient Y produce only a faint line. Had we used both the carcinoma of the cervix X and normal uterus Y at concentra-

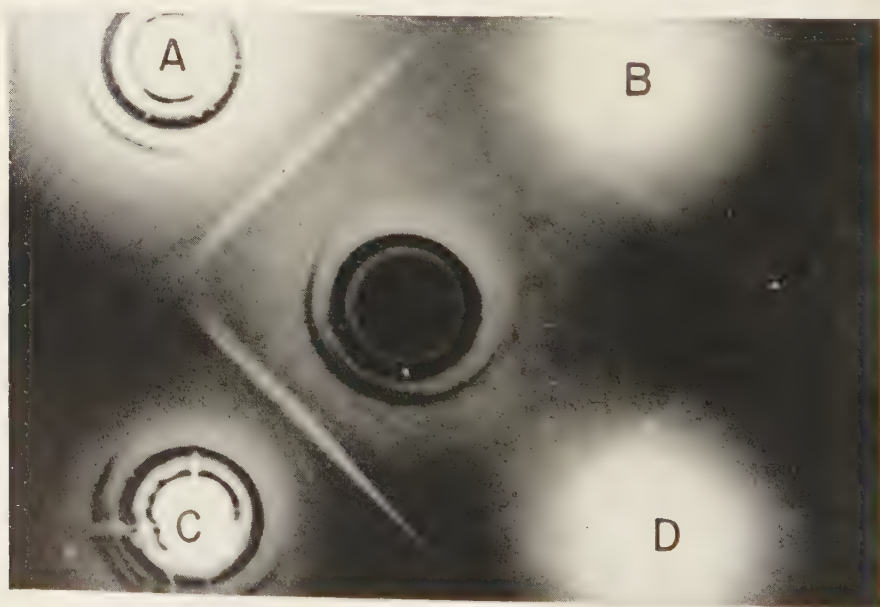


FIGURE 6. Center, antiovarian carcinoma No. 3 serum absorbed with plasma: (A) cervical carcinoma X (40 mg. ml.); (B) cervical carcinoma Y (40 mg./ml.); (C) normal uterus X (40 mg./ml.); (D) normal uterus Y (40 mg./ml.).

TABLE 1
THE DISTRIBUTION OF ANTIGEN 1¹⁵ IN SOME HUMAN TISSUES*

Present in	Absent from
4 Carcinomas of the cervix†	5 Normal uteri†
1 Carcinoma of the uterus†	3 Uterine fibromas
1 Normal uterus	5 Carcinomas of the ovary
	1 Normal ovary
2 Normal ovaries	1 Ovarian cyst
1 Ovarian cyst	4 Carcinomas of the breast
4 Carcinomas of the breast	1 Reticulum cell sarcoma‡
1 Reticulum cell sarcoma	
1 Normal spleen‡	
1 Normal stomach‡	

* An antiovarian cyst serum was used for the detection of this antigen, which was present in 50 per cent of all the tissues examined.

† Some of these tissues were from the same individual.

‡ Tissues from the same individual.

tions of 10 mg. ml. the carcinoma would still have reacted strongly, whereas no line would have been produced by the "control" tissue. Furthermore, the carcinoma could have been diluted at least fiftyfold without losing its reactivity. Consequently, if only these 2 tissues had been compared and the concentrations restricted to 10 mg. ml., we might have concluded that this antigen is "specific" for the tumor. This example emphasizes the necessity for using control tissues from the same patient. Furthermore, it illustrates the difficulties that one may encounter in the search for specific cancer antigens.

With the above limitations and reservations in mind, we shall now present some data obtained during a study of the distribution of several tissue antigens in over 40 surgical specimens.¹⁵ In this study 4 antisera capable of detecting 4 distinct antigens were used. Antigen 2 was present in all tissues and tumors, and antigens 3 and 4 could be detected in the majority of specimens. However, antigen 1 had a more limited distribution (TABLE 1). It was absent from 50 per cent of the specimens, including 5 carcinomas of the ovary. This finding is surprising, because the antigen could be found in normal ovarian tissue. It was also present in 4 cervical carcinomas and in a carcinoma of the uterus, but it was absent from most normal uterine tissues, even when they came from patients with carcinomas of the cervix or uterus. Antigen 1 was also absent from a reticulum cell sarcoma, although it was present in the spleen and stomach of the same patient, and in several other reticulum cell sarcomas. Consequently, it seems that antigen 1 is "group-specific" in that it is absent from 50 per cent of the population; superimposed on this is a degree of organ specificity, because the antigen cannot be found in some organs from the individual in whom this antigen is demonstrable.

The differences in antigenic composition of similar tumors are one of the most interesting features of our study. For example, most ovarian carcinomas contain at least 3 of the 4 antigens described in Korngold, 1956.¹⁵ However, one of these tumors, namely, ovarian carcinoma No. 4, lacked as many as 3 of

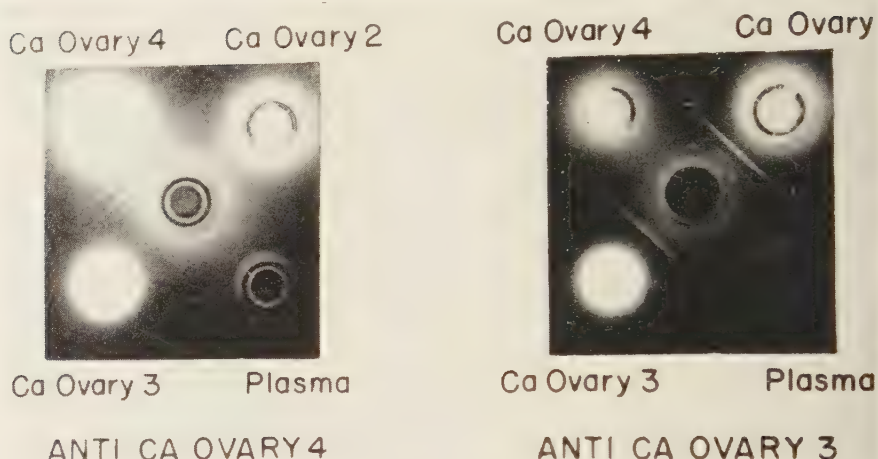


FIGURE 7. Antigenic differences among 3 ovarian carcinomas.

these antigens. Further antigenic differences became apparent when these tumors were studied with antisera prepared against them.¹⁶ An antiserum against ovarian carcinoma No. 4 produced 3 lines with the homologous tumor extract, 1 line with ovarian carcinoma No. 2, and no line with carcinoma No. 3 (FIGURE 7). The antiserum against carcinoma No. 3 did not react with carcinoma No. 4. The antisera could detect the antigens in the homologous tumor extracts in concentrations of 2 mg./ml. and 0.1 mg./ml., respectively.

The antiserum against carcinoma of the ovary No. 4 was relatively specific in that it reacted with only a few other tissues and produced 3 lines only with the homologous tumor. Furthermore, the distribution of some of the antigens in tissues from the same person varied. For example, when this antiserum was used to compare a carcinoma of the breast with the surrounding normal tissue, marked antigenic differences between these tissues became apparent (FIGURE 8). That the failure of the normal tissue extract to react with this antiserum cannot be attributed to faulty antigen extraction is apparent from the good reactivity of this extract with an antiserum against a carcinoma of the breast.

Considering all the complications and pitfalls that are inherent in the study of complex immunological systems, I should be foolhardy indeed to make, at this preliminary stage of our work, any claims as to the specificity of certain antigens. However, some antigens are present in relatively large amounts in certain tissues. Carcinomas very frequently may be distinguished from sarcomas on a quantitative basis. For example, in FIGURE 9¹⁶ reservoirs C and D contain 20 mg. ml. of extracts from two different sarcomas, and A and B contain extracts from two carcinomas. It may be seen that the carcinoma extracts contain an antigen that forms a line close to the antibody reservoir. This antigen cannot be detected in the sarcoma extracts when they are tested separately. However, in the presence of carcinoma extracts the small amount of this antigen in the sarcomas results in a deflection of the precipitin line of

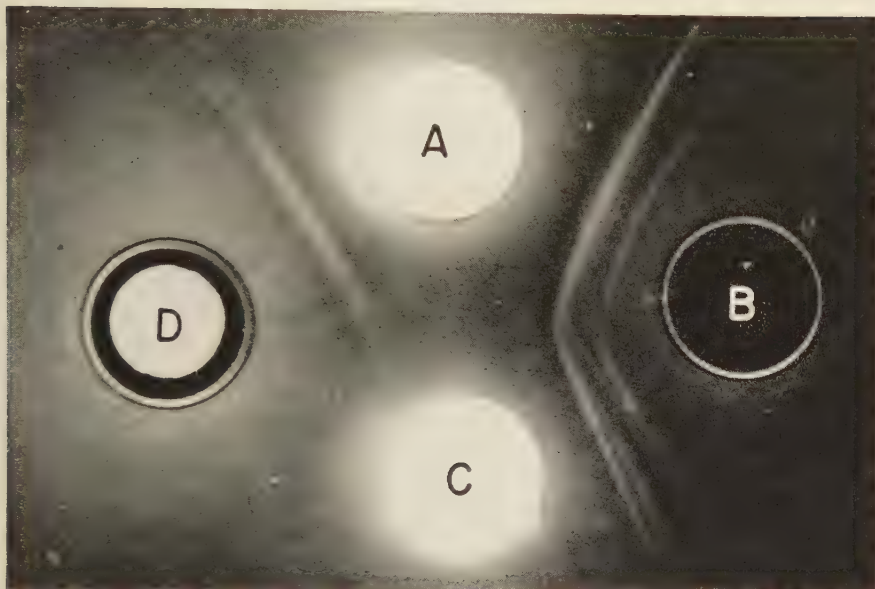


FIGURE 8. (A) Carcinoma of the breast (40 mg./ml.); (B) anticarcinoma of the breast serum absorbed with plasma; (C) normal breast tissue (40 mg./ml.); (D) antiovarian carcinoma No. 4 serum absorbed with plasma.

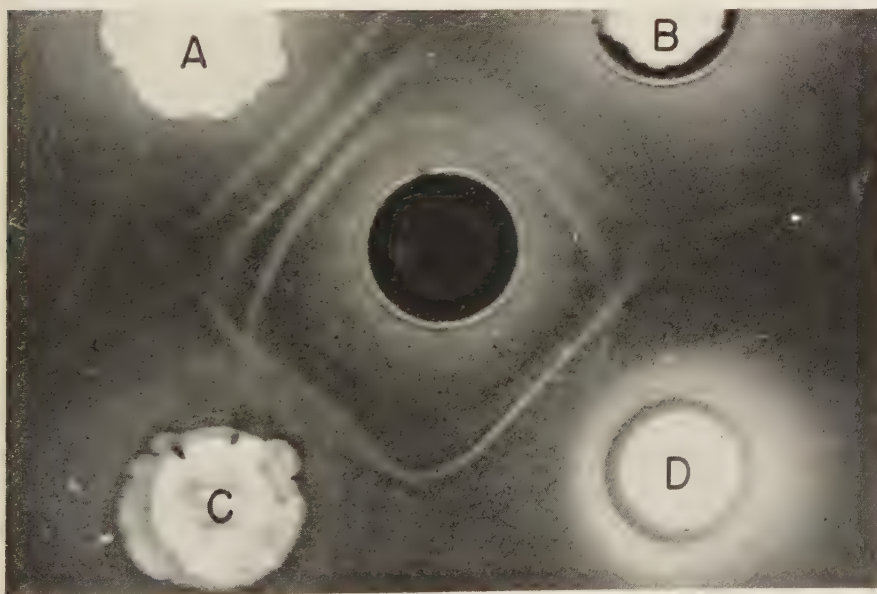


FIGURE 9. Center, carcinoma of the breast serum absorbed with plasma: (A) carcinoma of the breast (20 mg./ml.); (B) ovarian carcinoma No. 3 (20 mg./ml.); (C) sarcoma (20 mg./ml.); (D) sarcoma (20 mg./ml.).

the carcinomas. Of course, one may question whether the presence of small amounts of this antigen in the sarcoma extracts may not be attributed to contaminating epidermal cells or leukocytes.

The same picture shows that carcinoma of the breast contains one antigen that seems to be absent from the ovarian carcinoma, but this is a minor quantitative difference, because this antigen may be demonstrated in 40 mg./ml. of the ovarian carcinoma.

Most antitissue sera react with only a few of the many tissue antigens, suggesting that these antigens compete more successfully for the antibody-forming sites of the rabbit. If the limitation in number of antibodies is due to competition, it should be possible to induce antibody formation against some of the "poorer" antigens by their isolation and subsequent injection into rabbits. As a first step in testing this possibility, tumor and tissue extracts were fractionated by zone electrophoresis on starch.¹² Five different fractions were obtained that had electrophoretic mobilities corresponding to those of the serum proteins. These fractions were tested for their antigenic composition. In general, the fraction with the same mobility as serum γ -2-globulin contained few if any antigens capable of reacting with the antitissue sera; most of the tissue antigens detectable with our antisera are concentrated in the β - and α -2 fractions.¹⁷

Since both the slow and the very fast components of most tissue extracts failed to react with antisera against tissues and tumors, we decided to immunize several rabbits with these fractions made from a limited number of

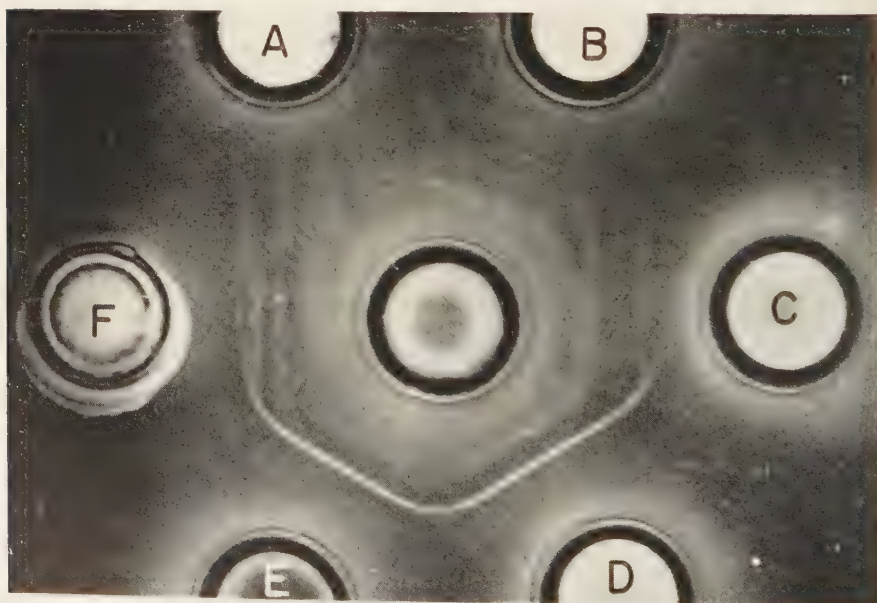


FIGURE 10. Center, antiserum against the fastest electrophoretic fraction of ovarian carcinoma No. 2. Around the periphery (A to E) are fractions of ovarian carcinoma No. 2, prepared by zone electrophoresis, at 10 mg./ml.: (A) γ -2 fraction; (B) γ -1 fraction; (C) β fraction; (D) α -2 fraction; (E) α -1 and albumin fraction; (F) ovarian carcinoma No. 2.

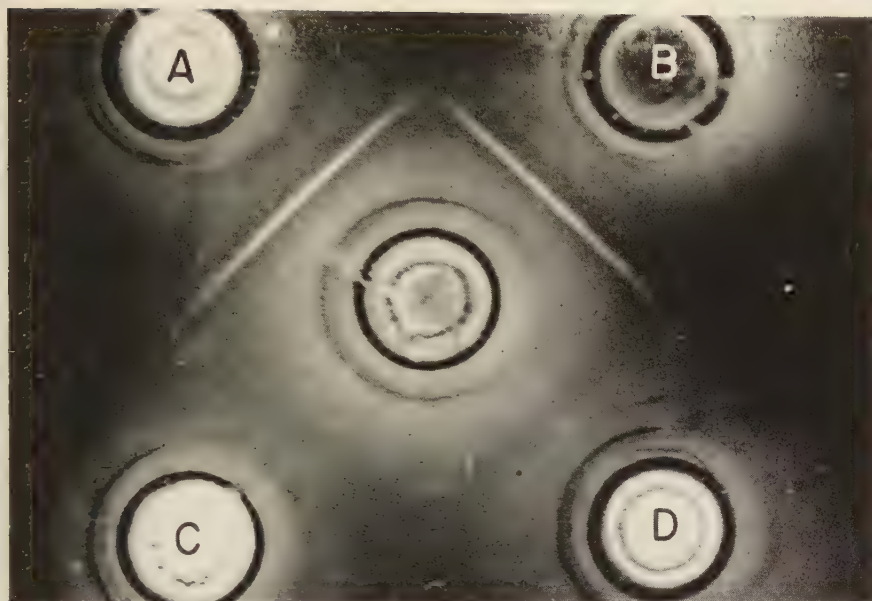


FIGURE 11. Center, antiovarian carcinoma No. 3 serum absorbed with plasma. Around the periphery (A to D) are fractions of ovarian carcinoma No. 3 prepared by ammonium sulfate fractionation: (A) 60 per cent saturation (6 mg./ml.); (B) 30 per cent saturation (10 mg./ml.); (C) saturated (10 mg./ml.); (D) 70 per cent saturation (12 mg./ml.).

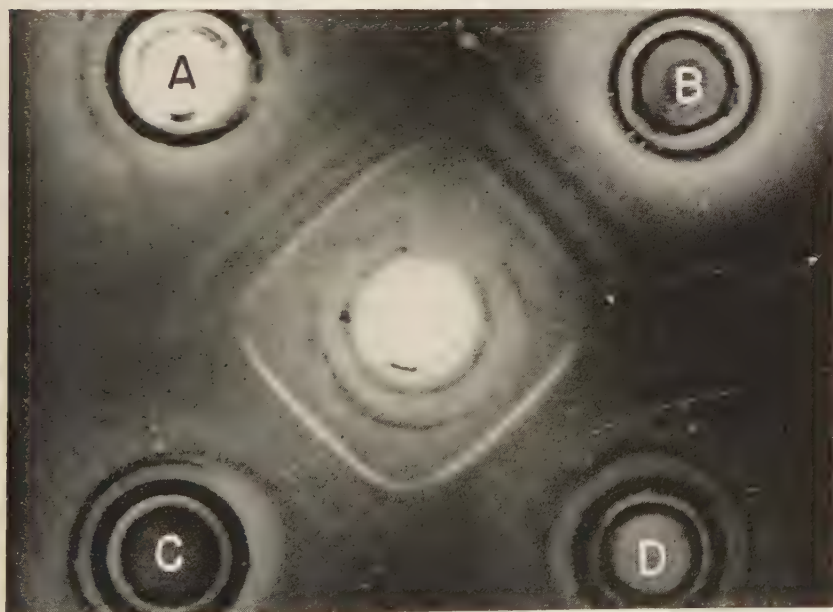


FIGURE 12. Center, antiovarian carcinoma No. 1 serum absorbed with plasma: (A) ovarian carcinoma No. 3 (20 mg./ml.); (B) ovarian carcinoma No. 3 (30 per cent ammonium sulfate—10 mg./ml.); (C) ovarian carcinoma No. 3 (60 per cent ammonium sulfate—3 mg./ml.); (D) ovarian carcinoma No. 3 (saturated ammonium sulfate—2 mg./ml.).

tissues and tumors. Potent antisera were obtained, showing that it is possible to produce antisera capable of reacting with weak tissue antigens. FIGURE 10 is representative of one of these antisera prepared against the fastest moving fraction of ovarian carcinoma No. 3. All fractions were used at concentrations of 10 mg. ml. The antigen responsible for the thick line is obviously most concentrated in the fractions with mobilities of α -2-globulin and albumin (D and E).¹⁶

Fractionation of tissue proteins with ammonium sulfate¹⁶ results in a partial separation of the antigens. Several of the antigens were concentrated to an extensive degree in the fraction precipitated at 70 to 100 per cent saturation, whereas others were more concentrated in the less soluble fractions (FIGURES 11 and 12). Spectrophotometric analysis of the fractions showed a shift of the absorption maximum from 280 $m\mu$ with the less soluble fraction to 260 $m\mu$ with the fraction precipitated at 70 to 100 per cent saturation (FIGURE 13). These results suggest that the latter fraction contains nucleic acids or purine and pyrimidine derivatives that are firmly bound to the proteins. These data, however, do not prove as yet that the antigens in this frac-

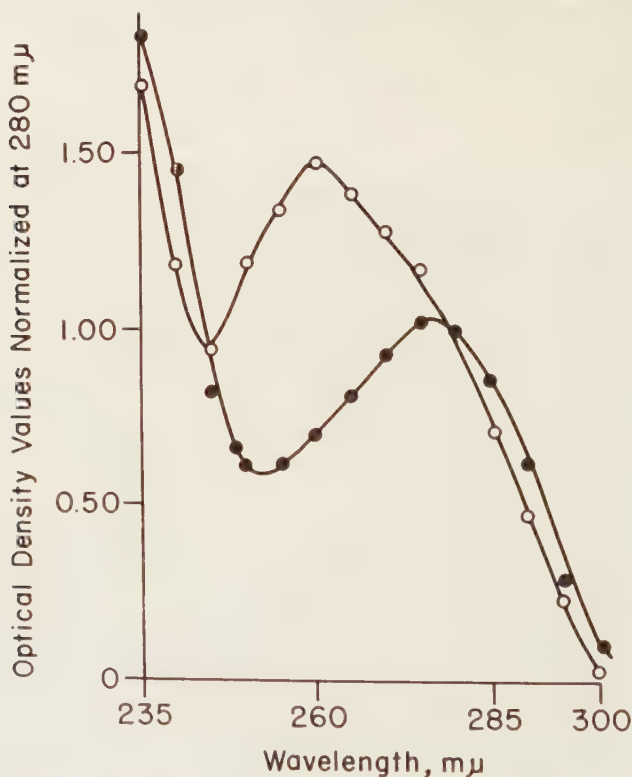


FIGURE 13. Absorption spectra of ovarian carcinoma No. 3 fractions obtained with ammonium sulfate.

Symbols: open circles = saturated ammonium sulfate; closed circles = 30 per cent ammonium sulfate.

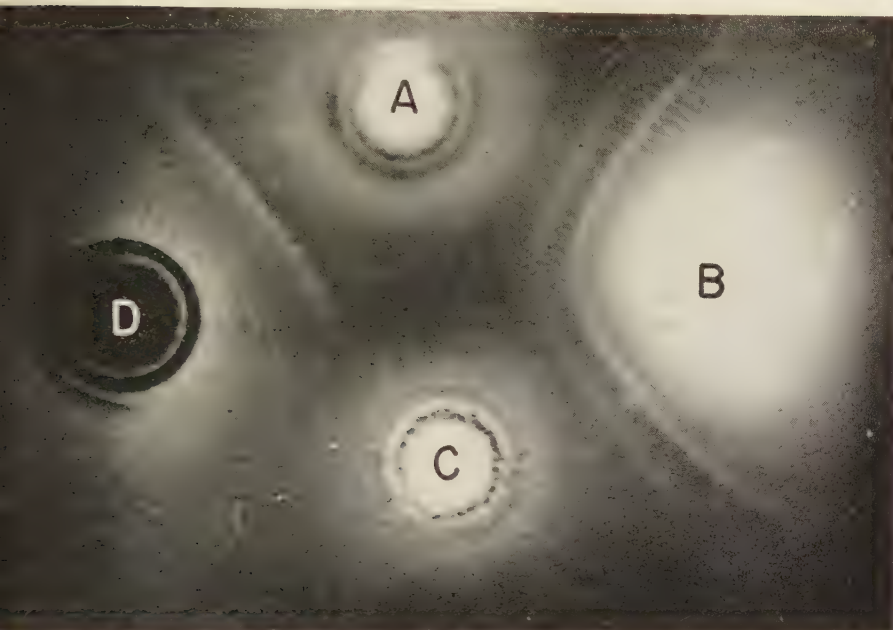


FIGURE 14. (A) Carcinoma of the breast (40 mg./ml.); (B) antiovarian carcinoma No. 1 serum absorbed with plasma; (C) carcinoma of the breast ($\frac{1}{2}$ hr. at 60° C.—40 mg./ml.); (D) antinormal uterus serum absorbed with plasma.

tion are nucleoproteins. Similar shifts in absorption maxima were observed with the fractions prepared by zone electrophoresis; the faster moving fractions have a maximum at $260\text{ m}\mu$, the slower one at $280\text{ m}\mu$.¹⁷

Many of the antigens studied were stable at 60° C. for 30 min., but at least one of them (antigen 3)¹⁵ was completely inactivated by this treatment (FIGURE 14).¹⁶

The objective of this study is to determine whether neoplasia results in, or is associated with, antigenic abnormalities. Such abnormalities may manifest themselves in three ways: (1) the tumor synthesizes an antigen that does not occur in normal tissue; (2) the tumor fails to produce an antigen present in normal tissue; and (3) the tumor synthesizes a protein that is related to the corresponding normal protein, but differs from it in certain respects. Of course, combinations of these three possibilities also may occur.

Attempts to prove either the first or the second of these are subject to great difficulties, since it is difficult to obtain from the same patient a tumor and a control tissue that are histologically and functionally similar, and it is almost impossible to prove that an antigen is actually absent.

Structural differences in a protein, however, are more easily determined by immunological methods. At present, we have shown that several forms of neoplasia result in abnormal protein synthesis, presumably because of a derangement of the synthetic mechanism in neoplastic cells.

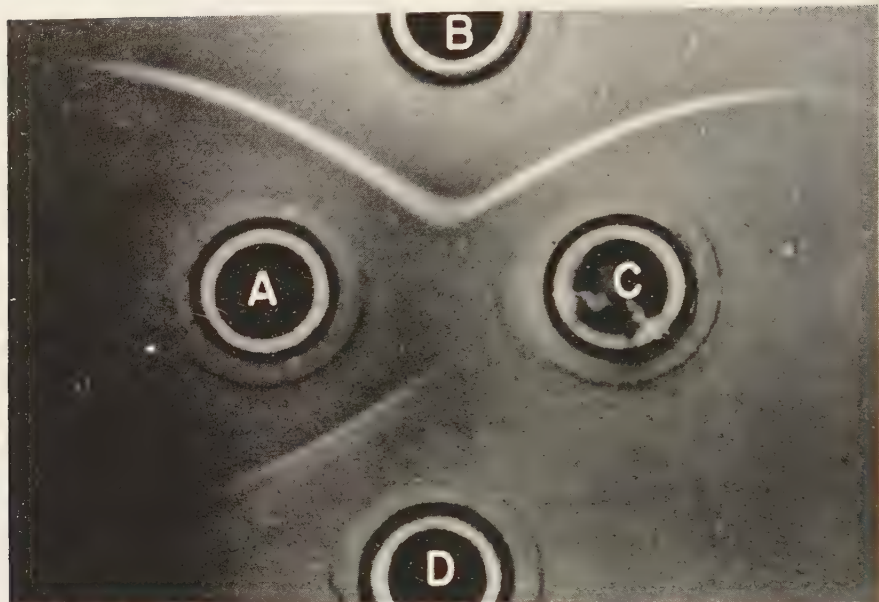


FIGURE 15. (A) Abnormal macroglobulin; (B) antiserum against abnormal macroglobulin; (C) normal serum; (D) antiserum against abnormal macroglobulin absorbed with normal macroglobulin.

In multiple myeloma the synthesis of the serum γ -2-globulin is altered to such a degree that the resulting proteins are abnormal antigenically¹² as well as with respect to their physicochemical properties.¹⁸ The abnormal globulins from patients with multiple myeloma are antigenically related to normal γ -2-globulin, but differ from it by lacking certain antigenic groupings.¹² Furthermore, they possess groupings that are specific for each patient's abnormal globulin.¹² The most radical deviation from normal protein synthesis is represented by the Bence Jones proteins, which seem to be incompletely synthesized abnormal serum proteins.¹⁹

Patients with macroglobulinemia synthesize large amounts of a macroglobulin that is found in small amounts in the γ -1-globulin fraction of normal serum. These pathological macroglobulins are related to the normal macroglobulins,²⁰ but lack certain antigenic groupings. The pathological macroglobulins differ among themselves, and antisera against such abnormal macroglobulins are specific for the homologous protein after absorption with normal macroglobulin (FIGURE 15).²¹ Our study of multiple myeloma and macroglobulinemia has resulted in a simple immunological test that distinguishes them from each other and from unrelated diseases that give rise to hyper- γ -globulinemia.²²

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INTERPRETATION OF HOST RESPONSE IN QUANTITATIVE STUDIES ON ANIMAL VIRUSES

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INTRODUCTION

Three different principles of biological assay have been applied to the measurement of biological activity of the animal viruses.¹ They are: (1) estimation of absolute numbers of virus particles by methods similar to those previously developed for the estimation of bacterial population densities; (2) determination of virus quantity in terms of "biological units," or as multiples of a unit quantity required to give some specific biological response in a specified population of test hosts; and (3) estimation of relative potency relationships among different virus preparations by reference to some common preparation that is designated as the "standard" (or control), and that is arbitrarily assigned the relative potency of unity, or 100 per cent.

The first two principles have been used widely by virologists and are well known to them. Analytical procedures pertaining to these two principles are therefore considered here only briefly as a background for a more detailed consideration of the third, which has found only limited use in the field of virology thus far.

Regardless of differences in type of biological response, or in principle of interpreting viral activity, all methods that have been developed for the bioassay of viruses have two things in common. First, they are all based upon the procedures of serial dilution and the testing of successive doses in a dilution series as a means of obtaining an "end point" response, or a graded or an enumeration response within some critical range. Second, the particular response, whether of the quantal, graded, or enumerative type, is highly correlated with dose (and therefore usable as a quantitative indicator of viral activity) only within a limited, critical dose range. The latter fact is, of course, the reason for the common procedure of serial dilution and, accordingly, for the general practice of considering virus dose on a logarithmic scale rather than on an arithmetic one in virus assays.

Advantage has been taken of these two common features as a means of orienting the following discussion of the interpretation of responses associated with various bioassay methods, particularly for: (1) illustrating the basic similarity of the analytical procedures that have been applied to different types of biological data; and (2) indicating the specific operational steps in the more general analytical method at which certain newer biometric tools, to be considered in some detail, may be applied to advantage.

ESTIMATION OF ABSOLUTE QUANTITIES OF VIRUS BY BIOLOGICAL METHODS

The two well-known methods for estimating numbers of virus particles from a dilution series are illustrated diagrammatically with respect to general methodology in FIGURE 1. In both charts of this figure theoretical dose-re-

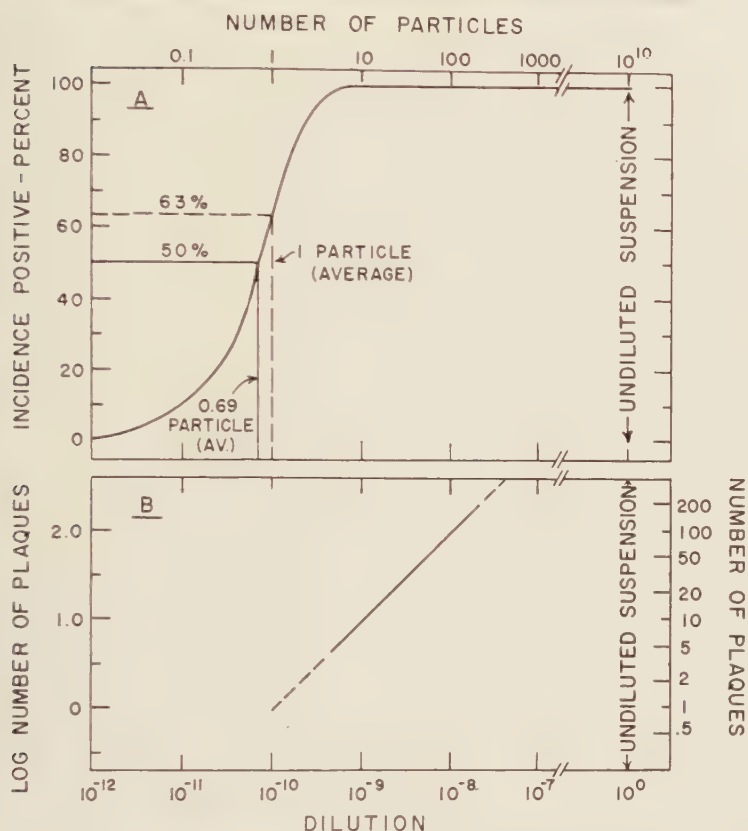


FIGURE 1. Theoretical dose-response curves based upon the Poisson distribution, illustrating the estimation of absolute numbers of particles in virus inocula.

sponse curves were constructed by use of the Poisson distribution for the chance variation of particles in unit (inoculum) volumes of a suspension. For the purpose of illustration the undiluted virus suspension was considered to have exactly 10^{10} particles, on an average, per unit (inoculum) volume. The assumptions were also made that: (1) the probability of a unit particle of virus coming into successful contact with a susceptible host cell (that is, the efficiency ratio) was 1:1, and (2) a single virus particle was capable of initiating a reaction leading to a detectable response. The dilution of virus that would be expected to contain exactly one particle per unit volume, on an average, is therefore 10^{-10} , by construction of the chart.

The upper chart of FIGURE 1 shows the S-shaped curve of percentage frequencies typically obtained when the biological response is of the quantal type (that is, the ratio of the number of individuals showing a positive reaction to the total number inoculated with the same dose). It is seen that the significant response range (that is, about 10 to 90 per cent) extends over about 1.5 log

intervals on the dilution scale. Since responses suitable for inferring absolute quantities of virus will follow a Poisson distribution, the number of particles per unit volume can be estimated readily from the percentage response, provided that one or more of the dilution groups gives a response within this significant range. In the upper curve, the percentage frequency expected (from the Poisson distribution) with inocula containing an average of exactly 1 particle (that is, 63 per cent positive and 37 per cent negative), has been made to coincide with dilution $10^{-1.0}$ (see above). Thus, it can be seen that the reciprocal of the dilution factor corresponding to the 63 per cent point interpolated on a Poisson curve (drawn through the observed percentage values within the significant range—see APPENDIX) gives an estimate of the absolute number of virus particles per unit volume used for the inoculations.

Because of its simplicity,² the method of Reed and Muench^{2, 3} is employed by most virologists for estimating the 50 per cent end-point dilution (ED₅₀), and the latter is used as the point of reference in interpretations of observed percentage data. Thus, as also illustrated in the upper chart of FIGURE 1, the reciprocal of ED₅₀, multiplied by the average number (0.69) of particles expected in inocula giving a 50 per cent response, yields an estimate of the number of particles per inoculum volume of the undiluted virus suspension.

The lower chart of FIGURE 1 represents the well-known dose-response relationship expected for the Poisson distribution when inocula, the same as above, are tested on solid biological surfaces that permit the development of plaques or lesions at sites of activity of individual virus particles.¹⁻⁴ This response also has a limited range, since too few particles per inoculum will yield inaccurate counts (unless large numbers of unit tests are carried out), and too many particles per inoculum will result in the coalescence of plaques or in an appreciable number of plaques that have been generated by more than one particle. Within the technically appropriate range, however, the average number of plaques produced by the unit inocula, multiplied by the reciprocal of the dilution factor, gives an estimate of the number of virus particles per unit volume of the undiluted suspension.

Since there is a direct proportionality between dose of virus (that is, the number of physical particles per inoculum) and the number of plaques, the curve relating dose and response will be linear and will have a slope of unity when both of the variates are plotted on the same scale, whether it be arithmetic or logarithmic. The test of closeness of fit of the observed results to a straight line with slope of unity is therefore a test of the fit of the mean counts to a Poisson distribution.

Since the doses in a dilution series represent equal increments on a logarithmic scale, it is the usual procedure to plot the number of plaques on a logarithmic scale also, in order to maintain a linear relationship, and thus to simplify interpretation of the data. This is an example of the principle of transformation of data to a function that simplifies interpretation and statistical analysis, a biometric device that is widely used in quantitative analyses of dose-response data of all types (see below). Wherever possible, we choose a function of response that will yield a straight line when plotted against the logarithm of dose. In the present example, therefore, the logarithm of the plaque count is

the appropriate transformation, or "response metameter," to be used with the logarithmic doses of virus.

Although in actual practice the ideal efficiency ratio of 1:1 is seldom exactly attained,⁴ the approach to this ratio is so close in certain virus-host systems⁴⁻⁷ that, for all practical purposes, the plaque count^{4, 5} or the 50 per cent dose^{6, 7} may be used as described for estimating absolute amounts of virus. Furthermore, absolute estimates (on a statistical average basis) should be possible even when the efficiency ratio is less than 1:1, provided that it is stable and that its average value is accurately known (see below).

ESTIMATION OF VIRUS IN BIOLOGICAL UNITS BY REFERENCE TO A STANDARD BIOLOGICAL RESPONSE

When the reactions of animals (or other biological test units) to a given virus are sufficiently stable, some criterion such as "50 per cent incidence" or other average result may be used as a standard response, and the quantity of virus (not necessarily known in absolute units) that causes this response may be considered as one biological unit. Such units have validity, of course, only for a given specified set of standardized test conditions, including the test hosts.

In many instances in the plant virus field,^{8, 9} and in the case of some highly adapted animal viruses,¹⁰⁻¹² the responses to a dilution series give an excellent fit to a Poisson distribution, even when the efficiency ratio is quite low or is unknown. The Poisson distribution therefore may be used, as in the preceding example, for interpretations with respect to "effective units" of virus. Unless the efficiency ratio is known and absolute estimates of virus quantity can be made, however, there is no particular advantage in using the quantity of virus giving the 63 per cent response as the unit of reference. Rather, it is more convenient to use as the biological unit the quantity of virus that yields a 50 per cent response because of the greater simplicity of methods for estimating this value.

FIGURE 2 shows dose-response curves based upon the Poisson distribution similar to those of FIGURE 1. However, they have been shifted to the right by 0.67 and 0.5 log units on the dilution scale, so that 1 biological unit in each case now falls exactly on dilution $10^{-9.5}$. Thus, if the concentration of virus in the undiluted suspension were considered to be 10^{10} , as in FIGURE 1, there would be 3.23 virus particles per ED50 unit, which means that the efficiency ratio would be 0.69:3.23 (= 1:4.7).

In the upper curve of FIGURE 2 the biological unit is defined as ED50 (that is, one standard inoculum volume of dilution $10^{-9.5}$). The titer of the undiluted suspension in biological units is, therefore, $10^{9.5} = 3.16 \times 10^9$ ED50.

In the lower curve of the same figure the biological unit is considered to be 1 pock-forming unit (PFU), or the quantity of virus required to produce exactly 1 lesion per standard inoculum, on an average, on the biological test surfaces (for example, chick embryo membranes or the leaves of plants). Thus, from the construction of the chart, the average pock or lesion counts at dilutions 10^{-9} , 10^{-8} , 10^{-7} , and so on, would be expected to be 3.16, 31.6, 316, and so on. Multiplication of any one of these counts by the reciprocal of the

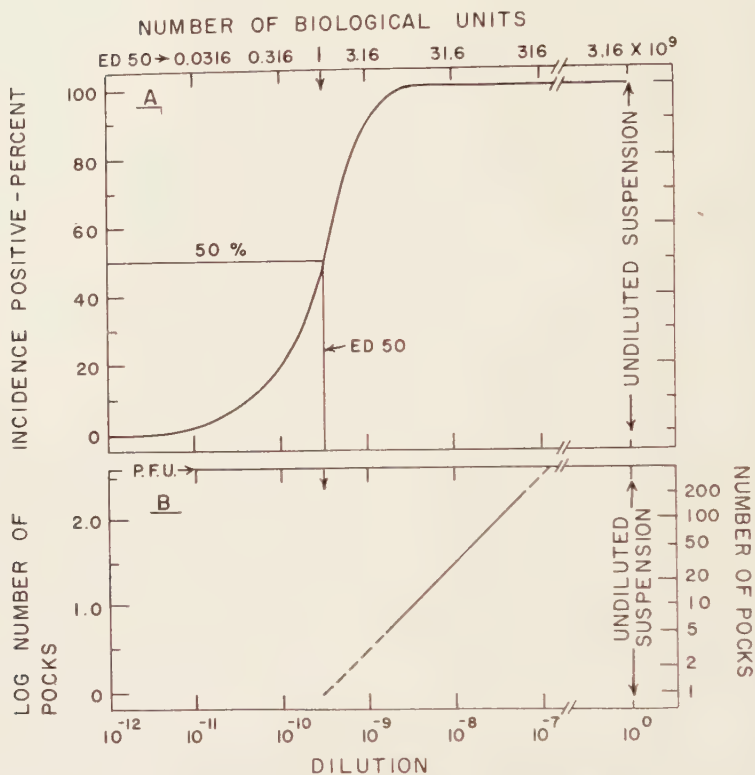


FIGURE 2. Curves similar to those of FIGURE 1 illustrating the estimation of virus quantity in terms of biological units.

corresponding dilution factor gives the titer of the undiluted suspension in biological units; that is, $10^{9.5} = 3.16 \times 10^9$ PFU.

FIGURE 3 shows some actual results (those of Parker,¹⁰ obtained with a highly adapted strain of vaccinia virus in rabbits) that give an excellent fit to a Poisson distribution. The observed percentage frequencies have been plotted on ordinary graph paper in the upper chart and on probability paper in the lower chart. The latter represents another biometric device for simplifying analyses, that is, by transforming S-shaped curves having the characteristics of an integrated normal distribution to a linear form. A further discussion of this transformation is given below. It is presented here to emphasize the fact demonstrated by both of the charts of FIGURE 3 that, even when the data fit a Poisson distribution, a normal distribution curve with a standard deviation (λ) of 0.5 (in log dose units) will also give a satisfactory approximation to the data within the significant response range. This is of importance to the present purpose, for which interpretations with respect to absolute numbers of particles are not concerned, because of the greater simplicity of methods based upon the normal distribution for estimating limits of bioassay error, significance of differences, and so on (see APPENDIX). Furthermore, there is no special ad-

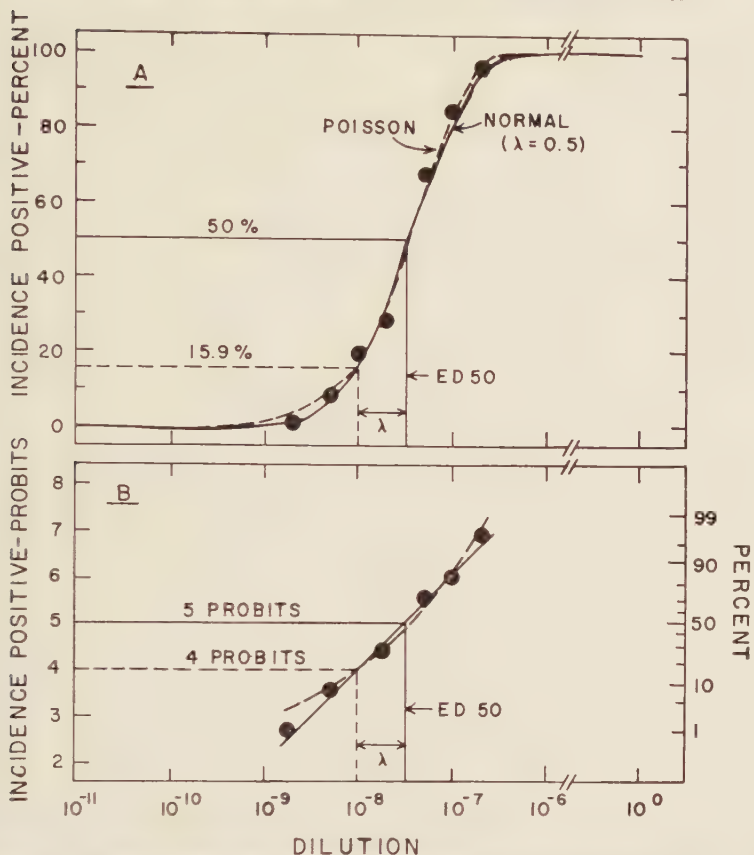


FIGURE 3. Showing approximations to actual animal virus data¹⁰ given by the Poisson "one particle" distribution and by an integrated normal distribution with standard deviation of 0.5 (in log dose units). (A) Plotted on ordinary graph paper; (B) plotted on probability paper.

vantage to the use of the Poisson distribution when the circumstances do not permit inferences with respect to absolute numbers of particles, particularly if, as is frequently the case with biological systems, the observed variance is greater than that expected theoretically from the Poisson equation.^{5, 11}

FIGURE 4 shows the percentage responses of chickens to serial tenfold dilutions of the Rous sarcoma virus.¹² In this instance it is clear that the Poisson distribution cannot be used for analysis of the data, but that they are fitted very well by an integrated normal curve with a standard deviation (λ) of 0.85 in log dose units. The fit of the percentage values to a straight line when plotted on probability paper, as in the lower chart of FIGURE 4, indicates that the results are adequately described by the normal distribution. This fact concerns primarily the choice of methods for estimating limits of error and for making tests of statistical significance (see APPENDIX). The determination of ED50 may be made by any appropriate method. For most biological studies

amounts of virus cannot be determined and when test hosts vary significantly from time to time in their responses to a virus, the only quantitative method for comparing results obtained at different times is through reference to a common ("standard") preparation of the virus. The comparison of results obtained at different times is, of course, dependent upon the availability of a stable preparation (for example, one preserved by freezing and storage at very low temperature²⁰). On the other hand, the same methods as are described for the estimation of relative potencies under these conditions also may be used for determining the relative potencies of any group of materials tested simultaneously, even when a stable standard is not available. In this instance an untreated, or "control," preparation may serve as the reference material of unit (or 100 per cent) potency.

The principle of comparing standard and unknown virus preparations is essentially the same as that employed in certain quantitative chemical analyses, such as those based upon colorimetric or turbidimetric procedures and involving the simultaneous comparison of standard and unknown samples of a chemical. In bioassays by this principle (see Irwin²¹ for an historical review) it is essential that the standard and unknown materials be tested in random groups of animals (or other test units) drawn from the same experimental lot, unless the population susceptibility is stable. When this is the case, the response to an unknown sample may be interpreted in terms of the quantity of standard virus that would be required to give exactly the same response under standardized conditions, as read from the standard dose-response curve. The principle is

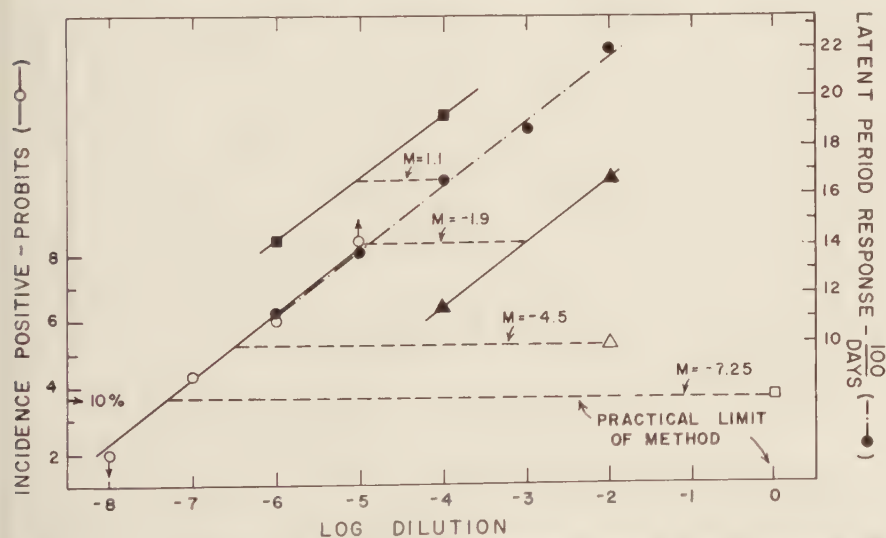


FIGURE 5. Illustrating the estimation of log potency ratios of virus preparations by reference to a curve for a standard preparation. The circles represent observed responses to a standard preparation of the Rous sarcoma virus. The other points represent assumed "unknowns" chosen for illustrating the procedure. The M values represent logarithms of the potency ratios of the respective unknowns, determined as horizontal deviations from the standard.

illustrated in FIGURE 5, in which the M values represent logarithms of potency ratios:

$$\begin{aligned} M &= \log (\text{potency of unknown}) - \log (\text{potency of standard}) \\ &= \log \left(\frac{\text{potency of unknown}}{\text{potency of standard}} \right) \end{aligned}$$

The antilog of M therefore represents the potency ratio in arithmetical terms. An alternate method of estimating M is that of determining the difference between responses at a given dilution (that is, the vertical difference on the graph), and dividing by the slope of the dose-response curve.

The central problem in bioassays by this principle is the same as that described for the other two, namely, the achievement of results within a limited critical range. The assays are therefore based upon the procedure of serial dilution as in the other instances. The particular dilutions to be inoculated and the particular design of the assay determinations will vary with the materials, the conditions, and the purposes of an experiment, as well as with the number of unknowns to be assayed at the same time.

The results diagramed in FIGURE 5 are based upon previously published¹² dose-response relationships and upon assumed "unknowns" selected for illustrating log potency ratios of different magnitudes. Since both tumor incidence (open points) and latent period (closed points) responses can be observed independently in the same group of animals, the results of both are charted on a single graph in order to emphasize the total dilution range available for bioassays of the Rous sarcoma virus with these two "biological indicators" (see Bryan, Calnan, and Maloney¹² for the actual experimental design that was followed in constructing the diagram).

Although it is not essential that the responses be in terms of a function that is linear with log dose for potency determinations to be made by this principle, the analyses are simplest when the relationship is linear. For example, the slope is constant (within the limits of experimental error) for similar virus preparations differing in potency, and log potency differences are represented by horizontal distances between parallel lines (or between points through which parallel dose-response curves are known to pass). Furthermore, statistical methods for estimating error terms are much simpler for linear than for non-linear dose-response relationships. The linear transformations of the responses represented in FIGURE 5 are discussed in the following section.

TYPES OF DOSE-RESPONSE DATA OBTAINED WITH VIRUSES

Any type of biological reaction that is quantitatively related to dose of virus may be used for estimating relative potencies by the methods described in the preceding section. The examples which follow represent actual data selected for the purpose of illustrating dose-response relationships of various general types, as well as the handling of certain problems that are encountered with some virus-host systems.

Quantal Responses

The percentage incidence of test animals showing an all-or-none reaction (for example, death or survival) is referred to in bioassay literature as a "quantal response." The S-shaped curves of the first four figures represent dose-response relationships for data of this type. When the S-shaped curve has the characteristics of an integrated normal curve it may be converted to a linear form by transforming the percentage values to probability units (probits) as illustrated in the lower half of FIGURE 4 (see FIGURE 11, below, for a diagrammatic representation of the principle of probit transformation). This is done either by plotting the observed percentage values directly on probability paper* or by converting them to probits for plotting on ordinary graph paper. Tables of probit values corresponding to various percentages at intervals of 0.1 are available in several publications on biometric procedures (see Bliss,¹⁴ Finney,¹⁶ Fisher and Yates,²² and Bliss and Calhoun²³).

In many virus-host systems the percentage responses are found to follow a normal distribution closely. Even in instances in which the integrated normal curve does not give an exact fit, the approximation to the data may be sufficiently close so that, for all practical purposes, the simplified methods applicable to the normal distribution may be used for obtaining approximate limits of error (see APPENDIX). An example of this was given above in the discussion of FIGURE 3.

For the purpose of comparing relative potencies by the principle discussed in the preceding section, the linear transformation of percentage data (that is, to probits) represents a general biometric device for analyses of such responses under all conditions, so long as the normal distribution gives a reasonably good approximation to the observed results. The relative potencies may then be determined in the same way as with any other type of response as illustrated in FIGURE 5.

Example 1. When the slope of the percentage-log dose curve is so steep that the entire critical response range (from 0 to 100 per cent) can be bracketed in the assay experiment, as illustrated in FIGURES 3 and 4, it may be more convenient to estimate log ED50 and to determine the log potency ratio (M) from the differences between log ED50 values. Thus,

$$M = \log (\text{ED50 for standard}) - \log (\text{ED50 for unknown}),$$

where ED50 is the *dilution* giving a 50 per cent response; or,

$$M = \log (\text{potency of unknown}) - \log (\text{potency of standard}),$$

where "potency" represents the titer, or the *reciprocal of the dilution*, and its logarithm is the cologarithm of ED50. This procedure is simply a special case of the general principle; that is, one in which the horizontal distance between dose-response curves is always measured at the 50 per cent (5 probit) level on

* A suitable type is that designed by J. Berkson and printed under the designation "No. 32,451. Normal Ruling" by the Codex Book Co., Inc., Norwood, Mass.

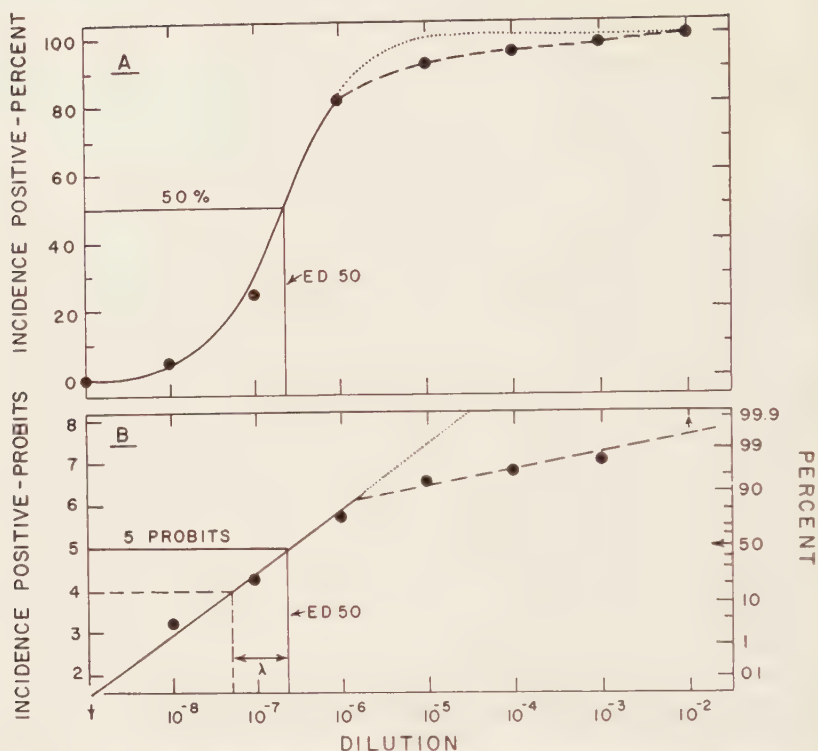


FIGURE 6. Showing truncation of the percentage responses due to the presence in the test group of a few aberrant, highly resistant individuals (data of Bryan¹³).

the response scale. Any convenient method for determining ED50, such as that of Reed and Muench,² may be used for comparisons at this constant response level.

The probit transformation provides an additional method for estimating log ED50 and its error. It is particularly useful where sufficient numbers of test animals are included in each dilution group so as to give regularity of the graphic results, because of the ease of determining log ED50, as well as the standard deviation of the distribution (λ), by the use of probability paper and by graphic interpolation. The procedures are illustrated in FIGURES 3, 4, 6, and 7. The estimate of log ED50 is simply the point on the log dilution scale corresponding to probit 5 (or 50 per cent), as read from the dose-response curve. The standard deviation (λ) in log dose units is the increment on the log dose scale corresponding to one probability unit on the response scale, that is, the increment between any two probit values. The use of this statistic for estimating errors of log ED50 and of M, as well as for judging the numbers of animals required for different degrees of accuracy, is discussed in the APPENDIX.

Example 2. A complicating factor in the interpretation of percentage responses obtained with some tumor viruses is the deviation of results at the

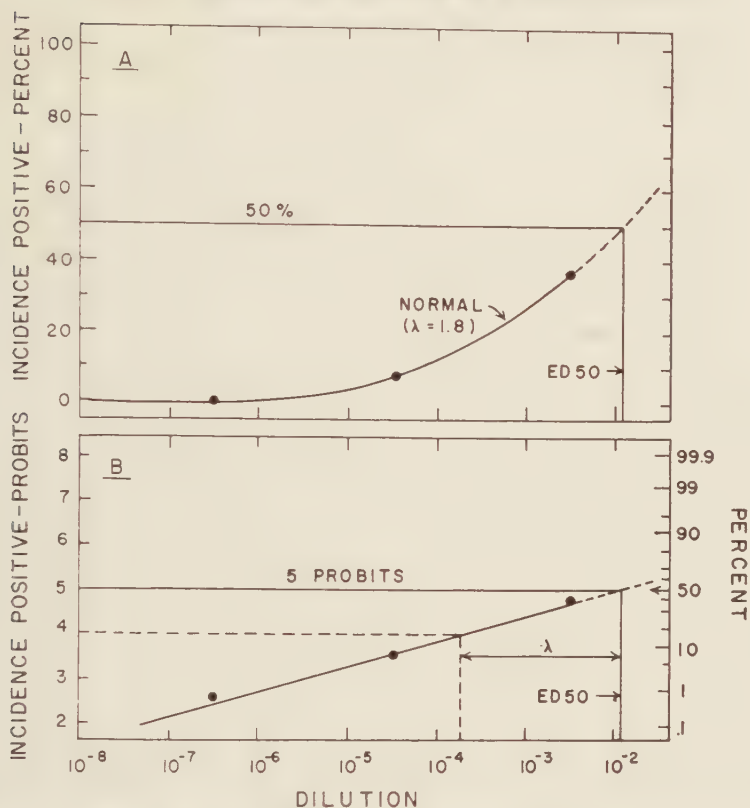


FIGURE 7. Showing truncation of the percentage responses due to a high natural resistance of the entire test group (data of Burmester²⁷).

higher percentage levels from any common mathematical model, including the Poisson and normal distributions (see data of Beard, Sharp, and Eckert,⁹ Bryan,¹³ Bryan and Beard,¹⁷ and Lo and Bang²¹). The deviations are due to the occurrence of a few highly refractory or totally resistant animals in a group that is otherwise fairly susceptible and uniform. The aberrant responses do not deviate in a regular or predictable fashion, but are highly variable and heterogeneous in successive experiments involving the same strain of hosts.

FIGURE 6 illustrates results of this type obtained with the Rous sarcoma virus.¹³ The upper curve shows deviations of the percentage responses above about 80 per cent, from the integrated normal curve drawn through the 50 per cent point as estimated by the procedure indicated in the lower chart. Following the lead of Eckert, Beard, and Beard,²⁵ who had applied the concept of the truncated normal distribution to the analysis of graded responses to viruses (see below), a similar interpretation was made of quantal response data of the present type, and there was proposed¹³ a method of graphic analysis that was patterned after that described by Bliss²⁶ for the analysis of truncated normal distributions involving graded responses.

The graphic procedure is illustrated in the lower chart of FIGURE 6, in which the percentages are plotted in probability units. It is clear that the results for the lower dilutions (higher concentrations of virus) follow a shallower slope than those for the higher dilution. The former are therefore considered to represent a biological truncation^{13, 25, 26} and are omitted from the analyses in deriving log ED₅₀ and λ .¹³

Example 3. In some virus-host systems the highest incidence of positive responses obtained with the strongest dose of virus may be less than 50 per cent, and the slope of the probit-log dose curve may be so shallow that comparisons at the 50 per cent level (which require extrapolation) may be less practical and less accurate than those made within the observed response range. In this case the probit equivalents of the observed percentage values may be employed for parallel line assays in the same manner as illustrated in FIGURE 5.

The data shown in FIGURE 7 have both of the above characteristics. They represent results obtained by Burmester²⁷ 63 days after the inoculation of lymphomatosis virus into young chicks. Although a higher incidence of lymphomatosis was observed at a later time (for example after 4 months), the slope of the dose-response curve was the same as earlier, and there was no appreciable gain in accuracy by prolonging the observation period for the assays by an additional 2 months. A similar shallow slope of the probit-log dose curve was observed by Eckert, Beard, and Beard²⁸ with the myeloblastosis virus in some strains of chickens.

Enumeration Responses

One of the earliest attempts to measure the biological activity of animal viruses was that of Calmette and Guérin²⁹ who, in 1901, made counts of the numbers of lesions produced on the skin of rabbits following inoculations with vaccinia virus. Burnet and Lush³⁰ and Keogh³¹ also described enumeration methods involving counts of local lesions or pocks produced on chorioallantoic membranes of developing chick embryos by the inoculation of various viruses. More recently, Dulbecco³² has developed a method of assay applicable to many animal viruses that is based upon the counting of plaques produced on monolayers of animal cells growing in tissue culture. In the latter instance and in the instance of some viruses tested on chorioallantoic membranes,^{11, 31} the counts have been observed to follow a linear curve with a slope of unity when plotted against dose and, therefore, to be consistent with a Poisson distribution. When this is the case, the lesion counts can be interpreted in absolute units where the efficiency ratio is known, or in biological units (PFU, for example) where it is not known, as described in earlier sections of this report.

In some virus-host systems, however, particularly in the plant virus field, the slope of the dose-count relationship is significantly less than unity, and the theoretical Poisson distribution cannot be used for potency determinations by these methods. An example of this is taken from the plant virus literature, since comparable data have not been found for any of the animal viruses, although they might be expected with some systems.

Example 4. FIGURE 8 shows some results that were published by Stanley³³ and used by Bald³⁴ in his consideration of bioassay procedures. These results

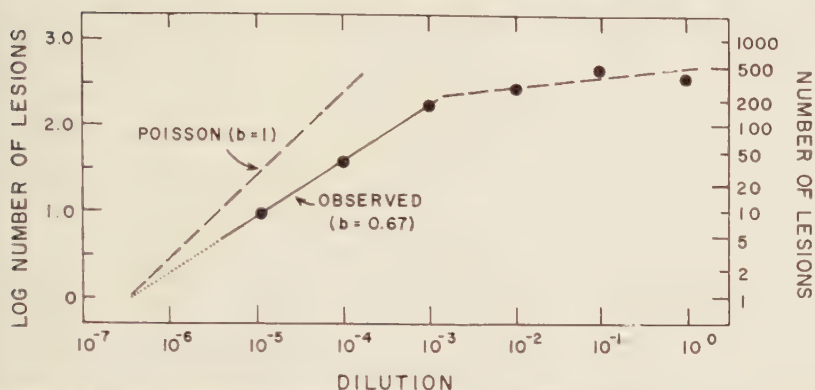


FIGURE 8. Showing the deviation of local lesion counts obtained with the tobacco mosaic virus (Stanley³³) from the expected curve for a Poisson distribution.

were obtained with resuspended pellet material recovered after ultracentrifugation of an infectious extract of plant leaves diseased with the tobacco mosaic virus. The problems associated with the bioassay of this virus were summarized by Stanley³⁵ in a review published in 1939. They include most of the problems that have been encountered in the bioassay of other viruses and, significantly, they were solved in Stanley's laboratory through the expedient of determining relative potency in relation to a standard curve. The latter was determined simultaneously with tests on the unknowns by use of a reference (or standard) preparation of the virus.

As illustrated in FIGURE 8, the lesion count is highly correlated with dose only within a limited critical range, and within this range the slope of the curve (0.67) is significantly less than that expected for a Poisson distribution (1.0). The product of the average count and the reciprocal of the dilution factor will not, therefore, yield the same titer in biological units at different dilution levels. In addition, the position of the curve for similar preparations was found by Stanley to vary appreciably from time to time in tests involving different lots of plants. Nevertheless, the logarithm of the count is linear with log dose within a range suitable for bioassay, and determinations of relative potencies by the principle illustrated in FIGURE 5 may be used for highly accurate quantitative studies on this agent (see Price, and Price and Spencer³⁶⁻⁴⁰ for further refinements of bioassay methods involving this principle as applied to plant viruses).

Graded Responses

The graded response most widely used in virus bioassays is the time interval between inoculation of virus and the appearance of a detectable response (for example, time to death and tumor latent period). Such graded reactions have been employed as biological indicators in bioassay procedures described for a number of viruses, particularly those that induce neoplasia. Since the following examples are taken from the tumor-virus field, the term "latent period" is employed in the general discussion, but it is intended to have a broader connotation that includes graded reactions in general.

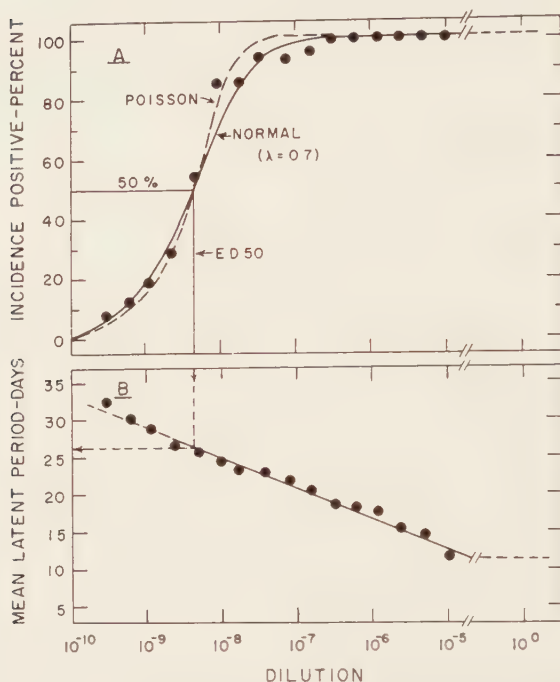


FIGURE 9. Showing responses at sites of inoculation of the Shope papilloma virus. (A) Percentage incidence of positive sites; (B) mean latent period of papilloma induction (data of Bryan and Beard⁴¹).

Example 5. The lower chart of FIGURE 9 represents an example in which the observed response (that is, the tumor latent period) was itself a linear function of the logarithm of dose and, therefore, usable directly for comparisons of relative potencies by the methods illustrated in FIGURE 5. The data are those published by Bryan and Beard⁴¹ showing the responses of rabbits to the Shope papilloma virus. A similar relationship was reported by Golub⁴² for the time-to-death of chick embryos following yolk-sac inoculations of the psittacosis virus (see APPENDIX for methods of estimating errors with data of this type).

The upper chart of FIGURE 9 shows the percentage responses of the same group of animals on which the latent period observations of the lower chart were made. The deviation of the higher percentage values from both the Poisson and the normal curve (drawn through ED50 as estimated by the method of Reed and Muench²) will be noted (see discussion above under *Quantal Responses*). At the time of publication of these data,⁴¹ the interpretation of such aberrant results as a biological truncation had not been made, and bioassays based upon the percentage response were considered to be less reliable than those based upon the latent-period response. However, application of the truncation procedure that was illustrated in FIGURE 6 should permit reliable bioassays to be made by use of the percentage responses in this instance also.

Example 6. For some virus-host systems the relation of the mean latent period in original time units (days, for example) to the logarithm of dose may

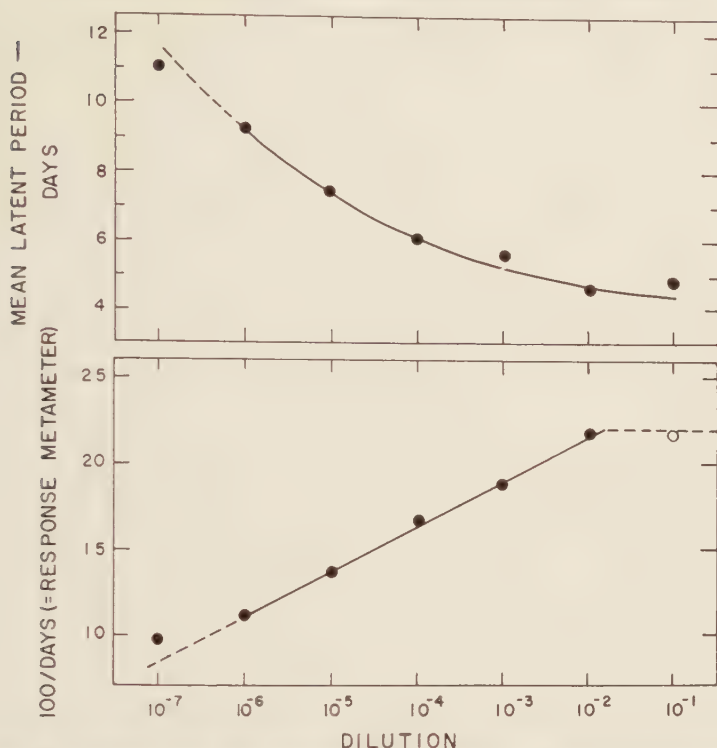


FIGURE 10. Showing the latent period of tumor induction by the Rous sarcoma virus. (A) Mean latent periods in original time units (days); (B) means of transformed latent period observations, in units of 100/days (data of Bryan, 1956¹³).

be curvilinear, instead of linear as in the preceding example. Such a relationship is illustrated in the upper chart of FIGURE 10, which represents the mean latent-period response of young chicks to subcutaneous inoculations of serial doses of the Rous sarcoma virus.¹³ In this case it is desirable to transform the original time measurements to some other function that will approximate a linear relationship when plotted against the logarithm of dose. As may be seen in the lower chart of FIGURE 10, the reciprocal of time in days yields a satisfactory approximation to a straight line. The reciprocals are multiplied by 100 in the graph in order to avoid decimal fractional values.

In other systems, such as myeloblastosis²⁵ and erythroblastosis¹² of fowls, the logarithm instead of the reciprocal of the latent period has been found to be the appropriate metameter for obtaining a linear relationship.

ANALYSIS OF GRADED RESPONSES WITHIN DOSE GROUPS

In the foregoing section several examples were given of the relationship of mean graded responses to the logarithm of dose. In certain instances it was necessary to transform the original units of measurement to some other function (such as the reciprocal or the logarithm) in order to obtain a function

(response metameter) that would follow a linear relationship when plotted against the logarithm of dose. The purpose of this transformation was to simplify the analytical procedures involved in estimating relative potency relationships.

In like manner, biometric devices involving the transformation of response units have been developed for simplifying the analysis of graded reaction data within dose groups, that is, within groups of animals (or other units) that have received exactly the same dose. The variations of such individual responses about their mean value are frequently asymmetric (or skewed) in terms of the original measurement units, and the frequency distribution may "tail out" toward the higher (or lower) measurement values. The former is particularly true for the latent period and for time-to-death data obtained with the tumor viruses. As is generally recognized, considerable skewness of the distribution of individual reactions can be tolerated in the comparison of mean values when the latter are based on very large numbers of observations. This is because the distribution of means based on large numbers approach a normal (symmetrical) distribution. On the other hand, the means of small numbers, such as are frequently used in bioassay dose groups, may be almost as badly skewed as the individual measurements. It is for this reason that the nature of the distribution within dose groups must be determined for each virus-host system if the most reliable procedures are to be used for expressing the mean result and its limits of error.

The first approach of biometricians to the problem of dealing with skewed distributions (see Ipsen¹³ for an historical discussion) was that of developing multiple mathematical models representing different types and degrees of skewness, and of selecting the one that most closely approximated a given set of observed data for use in the analysis of the latter. At best, the analysis of such skewed distributions is complex and highly laborious.

A more practical approach from the standpoint of routine bioassays is that based upon recent developments in biometric methodology, namely, the transformation of observed responses in a skewed distribution to some other function that will approach a normal curve in its frequency distribution. Thus, the conversion to their logarithms of units that tail out toward higher values has the effect of contracting the "tail" and of decreasing asymmetry. In some cases the log transformation may result in a frequency distribution that is essentially normal. So frequently has this been observed with graded biological data that the expression "log normally distributed" has become commonplace in biometric literature. In other instances, such as the latent-period response to Rous sarcoma virus, the function of response that is found to be normally distributed is the reciprocal, rather than the logarithm of the original units. It will be recalled that this was also the function that gave a linear relationship between mean response and log dose and that was employed as a basis for comparisons of potency in a preceding section. Fortunately, the same metameter usually provides both of these analytical desiderata, but this is not always the case, and it is sometimes necessary to use one metameter for determining relative potency and another for computing the error of the estimate of potency.

The following graphic procedures represent simplified biometric devices for selecting the function of a given response that best approximates a normal distribution, as well as for obtaining estimates of the mean and the standard deviation in terms of the transformed units. Although it may be preferable to derive the latter estimates by arithmetic procedures once the appropriate metameter units are known, the graphic determinations are simpler and more practical where there is biological truncation of the data (see below).

The Probit Transformation

The probit* transformation was originally devised for converting to a linear form S-shaped dose-response curves of the type obtained with quantal responses (see Finney¹⁶ for an historical review). In this instance the percentage values are determined from observed ratios and the results, as observed, follow a sigmoid curve when plotted against dose.

The same transformation also may be used for the analysis of graded responses *within dose groups*,²⁶ that is, for describing the variations of individual graded responses about their mean *at each successive dose level*. In this instance the original observations do not follow a sigmoid curve, but a frequency distribution that will be bell shaped when the distribution is normal. However, the results can be converted to a sigmoid form by accumulating the frequencies at successive measurement intervals. Such accumulative frequencies, expressed as percentages, then may be transformed to probability units in exactly the same way as was described for the quantal responses. In fact, the application to the quantal response is based upon the assumption that the observed sigmoid curve is identical with the curve that would be obtained if the minimum dose required to produce an effect could be measured accurately for each individual animal, and if the frequencies of such measured amounts were then accumulated and converted to percentages. A diagrammatic representation of the principle of probit transformation, independent of the actual nature of the measurement unit (that is, whether it is "time to produce an effect" or "just-effective dose") is presented in FIGURE 11. The three curves of this figure represent different ways of depicting the same variates in a normal distribution. They also show the relationship of probability units to the accumulative percentage frequencies on an S-shaped curve, and to the accumulative areas under a normal curve. As indicated at the right of the middle chart (B), probability units are simply the relative deviations in standard deviation units along the base of the bell-shaped curve (C) that have been transferred to the ordinate scale for use as alternate designations of the percentage responses, to which they bear a fixed relationship in a normal distribution. Normal deviates take + and - signs on either side of zero (the mean deviate). Probits were derived by C. I. Bliss by adding 5 (algebraically) to the normal deviate values to avoid the occurrence of differences in sign during computational procedures.

The detailed procedures for accumulating the frequencies and for estimating mean and standard deviation by the graphic probit method have been pre-

* A contraction of "probability unit."

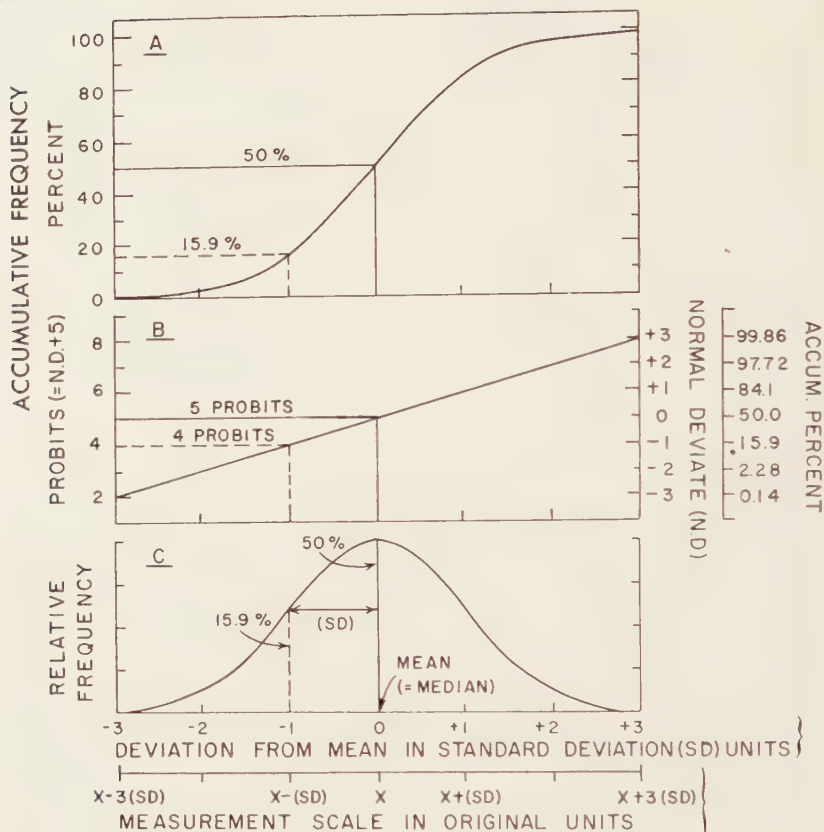


FIGURE 11. Showing different ways of depicting the variations in a normal distribution, and illustrating the derivation of probability units in the probit transformation. (A) Curve for accumulative frequencies, in per cent, as plotted on ordinary graph paper; (B) linear form of (A) obtained by plotting the accumulative percentages on probability paper, or by converting them to probits for plotting on ordinary graph paper; (C) curve for the variation of individual variates about their mean in a normal distribution: the accumulative areas under the normal curve, expressed as per cent, correspond to percentage frequencies on the upper S-shaped curve (A).

viously illustrated for homogeneous data.¹³⁻²⁶ The procedures are the same when there is biological truncation, except that only that part of the normal curve up to the point of truncation is used in deriving the mean and standard deviation. The probit analysis of graded responses is not considered satisfactory, however, when there are fewer than twenty-five¹³ or thirty²³ individuals in the dose group (see below).

FIGURES 12 and 13 illustrate the use of the probit transformation in testing the fit of observed latent periods to a normal curve, and in determining an appropriate function of time (time metameter) that will provide a reasonably good fit of the results to this distribution curve. They also illustrate the procedures for estimating mean and standard deviation when different types of biological truncation are involved.

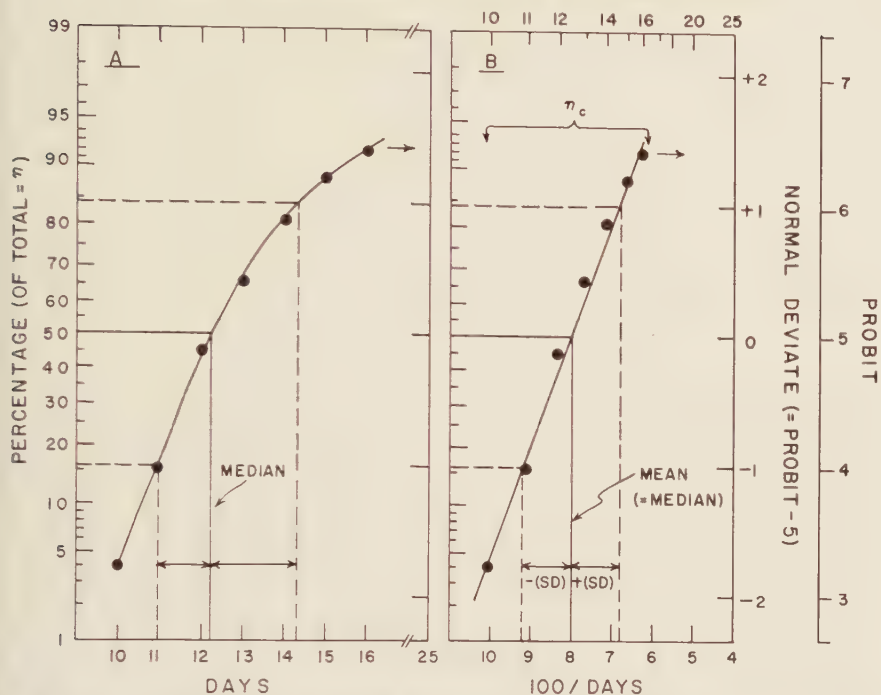


FIGURE 12. Illustrating the analysis of graded response data within dose groups by the probit method (data supplied by Vincent Groupé, Rutgers University, New Brunswick, N. J.).

FIGURE 12 represents the accumulative frequencies of time-to-death determinations in a group of 50 chickens that were inoculated intracerebrally with a constant dose of the Rous sarcoma virus.* Three of the 50 animals were highly refractory and failed to respond. The distribution is therefore truncated at 94 per cent. It will be seen that the results in original units (left chart) do not fit a straight line, and that the variation about the average (median) is asymmetrical. Transformation of the time units to their reciprocals ($\times 100$), however, resulted in a good fit to a straight line (right chart), indicating that the reciprocals are distributed essentially normally.

The data of FIGURE 13 represent latent-period responses reported by Eckert, Beard, and Beard²⁵ for the fowl myeloblastosis virus. The reciprocal of time in this instance did not give as good a fit to a straight line as did the logarithm. However, in some groups of chickens, as illustrated in FIGURE 13, the logarithmic function showed a break in slope after a certain time late in the observation period. The exact time of the break and the slope of the aberrant limb of the curve varied in a heterogeneous fashion among different lots of chickens and at different dose levels. This phenomenon was interpreted^{9, 26} as representing another type of biological truncation,^{13, 26, 41} possibly associated with

* Detailed data kindly supplied by Vincent Groupé of Rutgers University, New Brunswick, N. J.

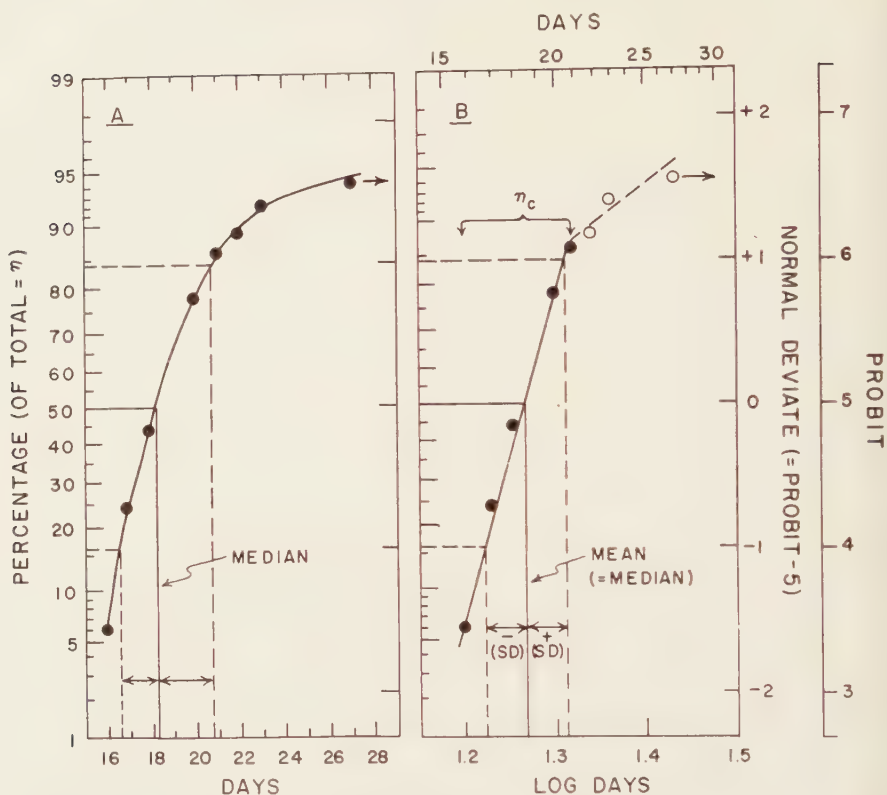


FIGURE 13. Illustrating the analysis of graded response data within dose groups when there is biological truncation due to the presence of aberrantly resistant individuals (data of Eckert, Beard, and Beard²⁵).

the development of an acquired partial immunity in some of the naturally less susceptible individuals as a result of the virus inoculations. The graphic procedures for estimating the mean and standard deviation (SD) in time metameter units are illustrated in FIGURES 12 and 13.

The Rankit Transformation

The term rankit* was introduced by Ipsen and Jerne,¹³ who applied to the analysis of graded response data the probability units known as "Scores for Ordinal (or Ranked) Data" that had been published by Fisher and Yates (see Table XX of their *Statistical Tables for Biological, Agricultural and Medical Research*²²).

The "scores" originally were devised for the analysis of nonmetric data, such as orders of preference in a psychological test, which nevertheless could be ranked with respect to some numerical category. Ipsen and Jerne¹³ showed the applicability of the same biometric procedures to the analysis of graded

* A contraction of "rank unit."

TABLE 1
SHOWING THE RANKIT TRANSFORMATION OF OBSERVED DEATH TIMES

Rank order of the observed result	Observed result		Rankit* ($n = 5$)
	Days	100, days	
1	50	2.00	1.16
2	39	2.56	.50
3	35	2.86	0
4	34	2.94	-0.50
5	28	3.57	-1.16

* Read from column for sample size 5 ($n = 5$) in published tables.^{22, 23, 43}

biological responses within dose groups, and published tables of rankits that were numerically the same as the scores of Fisher and Yates, except for rounding at the third instead of the second decimal. Similar tables have also been published by Bliss and Calhoun.²³

Rankits take values between -3 and $+3$, and are numerically the same as normal deviates (see FIGURE 11). They are derived, however, not as equivalents of percentage values on an accumulative frequency curve (as are probits and normal deviates), but as the average or "expected" values for different orders of rank of the observed results in a common series (or "sample") that has been arranged in order of the individual magnitudes. The numerical value of the rankit will vary for a given order of rank in accordance with the total number of ranked values in the series, that is, with the size of the sample. A different set of rankit values will therefore be obtained for each sample size or number (n) of animals in a group. In the tables cited above, a different set of rankit values is given for each sample size from $n = 2$ through $n = 25$,⁴³ $n = 30$,²³ and $n = 50$.²²

The assignment of rankit values to observed results is illustrated in TABLE 1, which represents the time-to-death of 5 chickens that had developed tumors following inoculations of the Rous sarcoma virus. Although the majority of the chickens in the group originally inoculated failed to develop tumors, the latter do not enter into the present analysis, since the response being considered is time-to-death of individuals with tumors, that is, the "tumor population" (a type of biological truncation that might occur in this population, however, would be failure to die as a result of the retrogression of a tumor). The times-to-death, in days, are arranged according to magnitude (that is, ranked in the second column), and in the third column the death times are given in terms of the candidate metameter units, 100 days. The rankit values of the fourth column were simply copied* for the respective rank orders from the appropriate column ($n = 5$) of published tables.

* Since rankit values are the same except for sign, on either side of zero, only half sets of rankit values were published by Ipsen and Jerne,⁴⁴ and, of "scores," by Fisher and Yates.²² Bliss and Calhoun²³ give only half sets for values of n greater than 10, although they include all rankits in their columns for sample sizes of 10 or less. Zeros also are omitted from such half-set tables, but they are to be inserted as the mid rankit of the group when the sample size is an odd number.

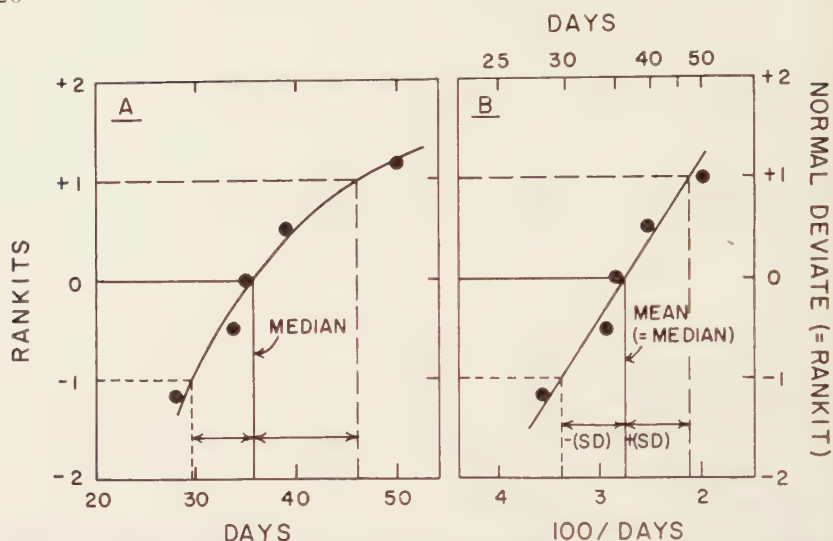


FIGURE 14. Illustrating the analysis of graded response data within dose groups by the method of rankits (see TABLES 1 and 2).

The use of rankits for testing the fit of the observed results to a normal distribution is illustrated in FIGURE 14. In the left chart the rankit values are curvilinear with time in days, but in the right chart they are seen to be linear with $100/\text{days}$. The latter units therefore may be considered to be distributed essentially normally and to be suitable for reliable biometric analyses of the data. It should be emphasized that this small group that is used to illustrate the procedure would be inadequate, alone, for deciding the appropriate metameter. However, once the metameter is determined through repetition of such tests or in a single test involving fairly large numbers of individuals, the rankit transformation may be used, as indicated in the chart at the right, for estimating mean and standard deviation.

When there are larger numbers of animals in a group, particularly when the response is a rapidly developing reaction such as the latent-period response to strong doses of Rous sarcoma virus, several of the individual animals may have identical response values. In this case the identical values are arranged in frequency groups as illustrated in TABLE 2, but the size of the sample used for determining rankits is still the total number in the group. The average rankit for the identical results is then used instead of the individual rankits for plotting against the time-interval values.²³

It will be noted that the times-to-response in units of $100/\text{days}$ are ranked in opposite orders in TABLES 1 and 2. The reverse order was used in TABLE 1 for comparison with the original units (days), which served as the basis for ranking. Although it is the usual procedure to represent individual responses greater than the mean by positive rankit values and those less than the mean by negative values, the analyses represented here (namely, the test of fit to a straight line and the estimation of the mean and the standard deviation) are

TABLE 2
ILLUSTRATING THE DERIVATION OF AVERAGE RANKIT EQUIVALENTS
WHEN 2 OR MORE OBSERVED RESULTS HAVE THE SAME VALUE
Latent-Period Data of Bryan, 1956¹³

Observed latent-period result 100/days	Frequency of individuals showing same result	Rank order of the 20 observations	Rankit* ($n = 20$)	
			Of individual results	Average for frequency group
18.3	5	1 through 5	1.87 through 0.75	1.22
15.5	13	6 through 18	0.59 through -1.13	-0.22
11.8	1	19	-1.41	-1.41
10.1	1	20	-1.87	-1.87
Total frequency (n)	20			

* Read from column for sample size 20 ($n = 20$) in published tables.^{22, 23, 43}

independent of the order of progression of the ranked observations (that is, of the signs of the rankits assigned to them).

The method of rankits is easy to use and, where biological truncation is encountered, it is an extremely valuable biometric tool for the analysis of graded response data within dose groups. It is particularly useful for the analysis of group results involving fewer than twenty-five⁴³ or thirty²³ animals, to which the method of probit analysis is not strictly applicable.^{23, 43} It should be applicable, also, to nonmetric data obtained with viruses, such as those represented by the classification of individuals into arbitrary categories (such as +, ++, +++, and ++++), according to the severity of their virus-induced lesions (see discussion relative to Table XX of Fisher and Yates²² for applications to data of this type).

DISCUSSION

An attempt has been made thus far in these pages to present the analytical procedures applicable to different types of virus-host systems in a manner that will permit them to be used by biologists having no special training in statistical methodology. Although based on statistical considerations, the graphic analytical procedures illustrated do not, in themselves, represent statistical operations. For example, the transformations of quantal data based upon the relationships of a normal curve (for example, to probits) have their basis in mathematical statistics, but in their applications they are analogous to, and no more difficult than, the transformation of arithmetic values to their logarithms. The further use of such transformed values is the same as if they were original observations (in suitable terms for simple and reliable analysis). Similarly, the application of probit or rankit analysis to graded response data is a means of finding a function of the original observations (for example, the logarithm or the reciprocal), which can be used for simple analysis of the data.

Where a sufficient number of test units such as commercial chick embryos, chicks, or noninbred mice can be used in each dose group to give regularity of the plotted results, highly accurate estimates of potency can be carried out by

the graphic procedures illustrated, without statistical analysis of the results. On the other hand, where the test units are highly expensive or in short supply, it may be necessary to use analytical statistical procedures for extracting the maximum amount of information from minimal numbers of animal responses, for example, by the use of iterative statistical procedures for fitting curves accurately to the more variable observed results. In any event, where there is biological truncation of the data (as is frequently the case with tumor viruses), it may be more practicable to use sufficient numbers of animals for reliable graphic analysis, where possible, than to carry out complicated statistical analyses of limited data.^{26, 43}

SUMMARY

Three different principles have been applied to the bioassay of animal viruses. The first represents the estimation of absolute numbers of virus particles from biological results by methods analogous to those employed for the determination of bacterial population densities. The second involves the determination of virus quantity in terms of biological units, a method generally employed where absolute particle counts are not feasible, but where the test hosts are stable in their average reaction to a virus. The third principle is that used with virus-host systems in which the average host susceptibility varies significantly from time to time, and to which, therefore, neither of the first two principles is applicable.

A general discussion is given of the interpretation of host responses in quantitative bioassays by all three principles. Specific analytical procedures applicable to the third principle, which thus far has found only limited use in virological research, are discussed in detail.

Simplified procedures for obtaining approximate estimates of experimental errors are summarized in the succeeding APPENDIX.

APPENDIX

A WORKING TABLE FOR USE IN THE ANALYSIS OF QUANTAL RESPONSE DATA THAT FOLLOW A POISSON DISTRIBUTION

When the percentage incidence responses in a dilution series consistently follow a Poisson distribution, the latter may be used as a mathematical model for analyses of the percentage results. Since it is the usual procedure in work with viruses to estimate the 50 per cent end-point dilution, log ED50, from the dilution data, the theoretical distribution curve may be superposed conveniently on the observed responses (that is, it may be fitted to the observed data) by plotting the 50 per cent point of the theoretical curve at this particular point on the log dilution scale. Other percentage values on the theoretical curve then may be plotted at successive increments on the log dilution scale by use of tables of exponential functions and the known relationship between dose (number of particles) and percentage response (the probability of the presence or absence of particles in unit volumes).

When the points on the log dilution scale are expressed as deviations (in log units) above and below log ED50, a working table can be prepared once and for all for plotting the corresponding percentages at these relative dilutions. Such a working table is shown in TABLE 3 for the theoretical Poisson distribution of one-or-more particles in unit volumes.

The zero value in Column 1 is log ED50 for the theoretical curve. In using the table, zero is superposed on the observed value of log ED50. The successive 0.3 log increments of Column 1, above and below zero, may then be counted off on the log dilution scale for plotting the corresponding percentages in Column 2. An alternate procedure is that of adding (algebraically) the observed value for log ED50 to each of the relative log values in the first column, and plotting the corresponding percentages of Column 2 against the resultant points

TABLE 3

THEORETICAL PERCENTAGES OF POSITIVE REACTIONS AND OF AVERAGE NUMBERS OF PARTICLES PER INOCULUM EXPECTED FROM THE POISSON ONE-OR-MORE PARTICLE DISTRIBUTION

Deviation from log ED50 in log units	Theoretical values	
	Per cent positive	Average number of particles
+1.2	(100)	11.1
+0.9	99.6	5.54
+0.6	93.8	2.77
+0.3	75.0	1.39
0	50.0	0.69
-0.3	29.3	0.35
-0.6	15.9	0.17
-0.9	8.3	0.087
-1.2	4.2	0.043
-1.5	2.2	0.022
-1.8	1.1	0.011

on the actual log dilution scale. The number of points given in the table is sufficient to enable one to draw a smooth theoretical curve visually.

The third column of TABLE 3 gives the theoretical values for numbers of particles per unit volume to be expected, on an average, when the efficiency ratio is known to be 1:1.

Analytical statistical procedures pertaining to the Poisson "one-particle" distribution in its application to virus bioassays have been discussed by Haldane⁴⁵ and Cornfield.⁴⁶ The analytical methods devised for estimating the most probable numbers of bacteria from the percentage results in a dilution series^{22, 47-51} also may be applied in estimating the most probable numbers of virus particles.

SIMPLIFIED PROCEDURES FOR DERIVING APPROXIMATE ESTIMATES OF STANDARD ERRORS FROM DOSE-RESPONSE DATA

The computation of exact limits of error from dose-response data requires considerable experience with statistical methodology. However, research virologists are not often concerned with critical tests of statistical significance nor with the testing of hypotheses that require exact estimates of error.⁵¹ An approximate error value will usually suffice, therefore, for determining the appropriate numbers of test animals (or of other biological units) needed to give the order of accuracy required for the purpose of a particular type of investigation.

The following methods are those that have been described⁵²⁻⁵⁴ for obtaining such useful approximations. For the most part, they represent minimal estimates of error or the limits below which the true errors surely do not fall. The numbers of animals predicted from them will also represent minimal values below which the actual required numbers certainly do not fall. Results that are not significantly different in comparison with these minimal limits of error are surely within limits of chance variation.

Standard Error of the Biological Unit

(1) *Log ED50*. The standard error of log ED50 may be determined approximately^{52, 53} by the following equation:

$$SE_{(\log ED50)} = \frac{\lambda}{\sqrt{\frac{n}{2}}} \quad (1)$$

where λ represents the standard deviation of the quantal responses in log dose units, and n is the number of animals in all dose groups within the response range from 7 to 93 per cent. The divisor 2 is used because quantal responses do not all have equal "weight" in determining log ED50, but an average weight of only 0.5 within the significant range 7 to 93 per cent, or 3.5 to 6.5 probits.^{52, 53} The value of λ may be determined graphically as illustrated in

FIGURES 3 and 4, or it may be computed as the reciprocal of the slope (b) of the probit-log dose curve; that is,

$$\lambda = 1/b$$

The following simplification of EQUATION 1 may be made for its general use:

$$SE_{(\log ED50)} = \frac{\sqrt{2}\lambda}{\sqrt{n}} = \frac{1.4\lambda}{\sqrt{n}} \quad (1a)$$

As was pointed out in the main text, a normal curve with $\lambda = 0.5$ gives a satisfactory approximation to the Poisson distribution for use in estimating the approximate error of log ED50. When the percentage responses are known to follow the Poisson distribution, therefore, EQUATION 1a may be further simplified to:

$$SE_{(\log ED50)} = \frac{1.4(0.5)}{\sqrt{n}} = \frac{0.7}{\sqrt{n}} \quad (1b)$$

When the probit-log dose curve is truncated, either as in FIGURE 6 or in FIGURE 7, the average weighting coefficient (0.5) cannot be used, and it is necessary to determine the actual weighting coefficients for the respective observed probit values by reference to published tables. The latter are included in the same publications that were cited in the main text for tables of probit values. The approximate standard error for such truncated distributions is then determined from the following equation:

$$SE_{(\log ED50)} = \frac{\lambda}{\sqrt{\Sigma(nw)}} \quad (1c)$$

where $\Sigma(nw)$ represents the sum of the products of the number of animals (n) and the weighting coefficient (w) for each of the dose responses (probit values) within the significant range.

(2) *Average number of biological units of virus per unit volume.* When the average lesion count produced by unit volumes is used for estimating the titer in biological units, the theoretical value of the standard deviation for the Poisson distribution may be determined from the known relationship between the mean (\bar{x}) and the standard deviation (SD); namely,

$$(SD) = \sqrt{\bar{x}}$$

The minimal value (that is, under ideal conditions) of the standard error ($SE_{\bar{x}}$) of a mean count will therefore be given by

$$SE_{\bar{x}} = \sqrt{\frac{\bar{x}}{n}} = \frac{(SD)}{\sqrt{n}} \quad (2)$$

where n represents the number of replicates (for example, the number of different membranes on which separate counts were made), or by

$$SE_{\bar{x}} = \frac{x}{\sqrt{N}} \quad (2a)$$

where N represents the total number of lesions counted on all replicates.

Standard Error of the Log Potency Ratio (M)

(1) *Quantal responses.* When the log potency ratio (M) is determined from quantal responses, the individual animal responses have an average weight of only 0.5, as in the determination of log ED50. If M has been estimated as the difference between two log ED50 values (that is, for standard and unknown, see main text), and the standard errors of the latter already have been determined, the standard error of the difference (SE_M) is given by

$$SE_M = \sqrt{(SE_{50})_s^2 + (SE_{50})_u^2} \quad (3)$$

where $(SE_{50})_s$ and $(SE_{50})_u$ represent the standard errors of log ED50 for standard and unknown, respectively.

If the standard errors of the log ED50 values have not already been computed, the following form of the same equation may be used.

$$SE_M = \lambda \sqrt{\frac{\frac{\lambda_s^2}{\left(\frac{n_s}{2}\right)} + \frac{\lambda_u^2}{\left(\frac{n_u}{2}\right)}}}{\quad (3a)}$$

where the subscripts s and u represent the respective statistics for standard and unknown.

When the value of λ is the same for both materials (that is, when the probit-log dose curves are known to be parallel), equation (3a) may be simplified to

$$SE_M = \lambda \sqrt{\frac{2}{n_s} + \frac{2}{n_u}} \quad (3b)$$

and, when the same number (n) of animals is included within the significant range for both the standard and the unknown, a further simplification may be made to

$$SE_M = \frac{\sqrt{8}\lambda}{\sqrt{N}} = \frac{2.8\lambda}{\sqrt{N}} \quad (3c)$$

where N represents the total number within the significant ranges for *both* the standard and the unknown (that is, $N = 2n$).

The latter form (3c) of the equation is that most conveniently used for predicting approximate standard errors of log potency ratios on the assumption that the assay will involve equal numbers of animals in each of the two materials. The rearrangement of the latter equation, which solves for N , is therefore the most convenient form for predicting the number of animals required for a given level of accuracy⁶⁴, that is,

$$N = \frac{8\lambda^2}{(SE_M)^2} \quad (3d)$$

where SE_M is assigned the standard error value, in log units, desired for the assay. If it is desired to estimate the number of animals required to give a "maximal" error (that is $2 \times SE_M$) of a given size, the value entered in the denominator is $\left(\frac{SE_M}{2}\right)^2$.

When the probit log dose curves are parallel, the above equations may be used for estimating approximate SE_M , even when the potency comparison does not involve ED50 determinations (that is, when M is determined as the distance between parallel lines). However, in all instances in which the average value, 0.5, is used as a weighting coefficient, the observed responses within the significant range must be approximately symmetrical on either side of the 50 per cent response.

(2) *Graded responses.* When the log potency ratio (M) is estimated from responses of the graded type, and when the standard deviation is the same (within limits of chance variation) for all dose levels within the significant range, the individual animal responses will have equal weight in determining the standard error of M . The approximate standard error of M under these conditions is given by

$$SE_M = \frac{2\lambda}{\sqrt{N}} \quad (4)$$

where N , as above, is the total number of animals within the significant ranges for *both* the standard and the unknown, and where λ is the standard deviation in log dose units, derived as described below.

The rearrangement of EQUATION 4 used for estimating numbers of animals (N) required for a given accuracy, is

$$N = \frac{4\lambda^2}{(SE_M)^2} \quad (4a)$$

If the standard deviation (SD) varies at different dose levels within the significant range, the individual responses will not have equal weight, and weighting coefficients will have to

be used in a manner similar to that described for the quantal responses of the preceding section. The weighting coefficient is derived in this instance as the reciprocal of the variance (that is, the square of the standard deviation) at each dose level (see Bryan and Shimkin⁵⁵ for an illustration of the weighting procedure). A rough approximation will be given, however, by use of the average standard deviation for the entire dose series. Usually, a response metameter can be found which will have a constant standard deviation for all dose levels, and it is preferable to transform the data to such units for the estimation of errors.

Standard deviation (SD) in response metameter units. The standard deviation (SD) of the responses within each dose group may be determined by the graphic procedure illustrated in FIGURES 12, 13, and 14, or by the computational procedures generally employed for estimating standard deviation (see any text book on statistical methods involving small numbers). In the latter instance it is essential that a response metameter that approximates a normal distribution be employed for the estimation of errors of M by the present approximate methods.

Standard deviation (λ) in log dose units. As in the discussion of quantal responses, λ is used to denote the standard deviation of individual responses in log dose units, that is, the equivalents of (SD) in terms of individual inferred-potency values. In the case of graded responses, λ represents the ratio of the average standard deviation of the observed responses to the slope of the log dose-response curve;⁵⁶ that is,

$$\lambda = \frac{(\overline{SD})}{b} \quad (5)$$

where (\overline{SD}) is the average value of (SD) determined for the separate dose levels, and b is the regression coefficient, or slope, of the log dose-response curve. The slope may be determined graphically by counting off the number of units on the response scale corresponding to 1.0 unit (1.0 log interval) on the dose scale, as read from the dose-response curve, or it may be determined by taking any two points (x_1y_1 and x_2y_2) on the curve, and substituting in the following equation:

$$b = \frac{y_1 - y_2}{x_1 - x_2} \quad (5a)$$

A nomogram was previously published (see text and Figure 3 of Bryan,⁵⁷) for determining minimal values of SE_M or N , by the relationship expressed in EQUATION 4, when the value of λ is known. A table was also given (Table 1 of Bryan⁵⁷) that showed the numbers of animals (or other units) corresponding to various "maximal" limits of error (that is, $2 \times SE_M$) for different levels of the "precision index," λ . Both the nomogram and table were constructed on the assumption that each biological test unit would have equal weight in determining M . However, as was pointed out in the original publication,⁵⁷ the nomogram may also be used for other systems in which the weights are not equal by correcting the number (N) according to the average value of the weighting coefficient (that is, by dividing N by the average value of the coefficient). For example, the quantal responses discussed above had an average weighting coefficient of 0.5, and the corrected values of N would in this instance be twice those shown in the nomogram of Bryan.⁵⁷

A detailed discussion of analytical procedures pertaining to graded responses similar to those considered here has recently been given by Prince, Littell, and Ginsberg⁵⁸ in their description of methods applicable to the bioassay of viable tumor cells.

THE USE OF TIME-FREQUENCY CURVES FOR STUDIES ON HOST RESPONSE TO CONSTANT DOSES OF VIRUS

In addition to representing convenient curves of errors for the analysis of variations of individual graded responses within dose groups (see preceding section), transformed time-frequency curves such as those of FIGURES 12 to 14 also represent useful devices for the presentation of results obtained with different experimental groups of animals treated with the same dose of virus. For example, in studies of factors that influence host response to a virus it is frequently desired to standardize the virus dose and to test the effects of various experimental treatments or conditions on the responses at this constant dose level. When the time-frequency data are plotted in probits or rankits against a time metameter that yields a straight line, the resultant curves allow visualization of the spread, or of deviations, of the individual reactions as well as of the respective graphic positions of the group average responses. The use of this method for the presentation of data obtained with the Rous sarcoma virus has been illustrated by Groupe *et al.*^{59, 60}

When the standard deviation (SD) is computed from the data in appropriate metameter units, the result is an accurate rather than an approximate determination of this statistic for the variations *within* the dose group.* The standard error ($SE_{\bar{y}}$) of the mean response (\bar{y}) given by the usual formula for the standard error of a mean is also an accurate estimate; that is,

$$SE_{\bar{y}} = \frac{(SD)}{\sqrt{n}} \quad (6)$$

where n represents the number of individuals in the dose group. Accurate inferences with regard to statistical significance may therefore be made between the responses to different experimental groups that have been treated with the same dose of virus.

Data plotted as illustrated in FIGURES 12 to 14 allow visualization of the standard deviation, since the response is expressed in probability units. Furthermore, if the numbers included in the various groups are given, determinations of statistical significance can be readily made, by anyone so interested, from the graphic estimates read from the chart.

When a sufficient number of animals is used in each group so that a straight line can be drawn accurately through the plotted points by sight, the graphic estimate of SD will also be an accurate value. If the data are truncated, however (see FIGURES 12 and 13), the corrected number (n_c) of animals in the group (that is, those included up to the point of truncation of the curve) is used in determining the standard error of the mean; that is,

$$SE_{\bar{y}} = \frac{(SD)}{\sqrt{n_c}} \quad (6a)$$

See Bliss²² for a comprehensive discussion of the graphic analysis of time-frequency data by this method, and Bliss²² and Ipsen²³ for computational procedures to be used in correcting for truncation.

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* On the other hand, λ and the error of the log potency ratio (SE_M) determined from (SD) are approximate estimates only, since the contribution of the error of the slope (SE_b) of the dose-response curve to the final error is neglected in the approximate procedures described herein (see preceding section).

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VIRUS NEUTRALIZATION*

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The study of the tumor viruses from the immunological point of view has been limited in scope. In the course of an investigation of the properties of the virus of avian myeloblastic leukosis it was essential to approach one aspect of the immunology of this virus, namely, an antigenic analysis of the virus particle. The value of such an analysis was manifold, including the application of serologic evidence to the definition of the homogeneity of the virus materials, the preparation of specific antisera for the classification of the tumor viruses and their variants, and the establishment of the basic data and concepts on which could be constructed an understanding of the role of immunity in the infectious processes of this group of viruses.

At this time when the viral etiology of a number of different tumors is being elucidated and the study of these agents passes from the descriptive to the experimental stage, it is worthwhile to review some aspects of the immunological characterization of the virus of avian myeloblastic leukosis. This investigation of one of the complex of leukosis viruses is particularly informative in illustrating some of the requirements that must be met if the methods of antigen analysis are to be applied to other tumor viruses.

First, it is proper to summarize the essential properties of the virus.² Passage of the virus is accomplished by intravenous inoculation of filtered infectious plasma into 3-day-old chicks of a special inbred line. If the dose is adequate, virtually all chickens will develop myeloblastosis within 10 to 30 days. The plasmas of some of these chickens will contain as many as 10^{12} virus particles per ml. The appearance of such plasmas in the electron microscope indicates a relatively uniform population of virus particles of an average diameter of $110\text{ m}\mu$.¹⁶ The uniformity of the suspensions of virus particles in a simple plasma milieu and in virus concentrates makes it possible to count the number of virus particles in a sample with a high degree of precision.¹⁸ Another important characteristic of the virus is the adenosine triphosphatase (ATPase) activity, an enzymatic activity related quantitatively to the virus particle itself.¹⁵ Finally, a quantitative and reproducible method of bioassay of the infectivity of the virus samples is available.⁸ The precision index³ of this method is 0.6, so that, using the adequate number of animals employed in these experiments, it was possible to measure the relative infectivities of the samples with an error of 30 per cent or less.

Critique of the Methods

Evaluating this investigation in terms of its contribution to the understanding of the tumor viruses as a group, we see that there is as much to be gained

* The experimental results reported in this paper were obtained while the author was a Scholar in Cancer Research of the American Cancer Society, Inc., New York, N. Y. They constitute, in part, conclusions reached in an investigation of the group of leukosis viruses made in the Division of Experimental Surgery, Department of Surgery, Duke University School of Medicine, Durham, N. C., under the general direction of J. W. Beard.

from a discussion of the methods as of the results themselves. Seen in retrospect, the possibilities and limitations of the various methods and the nature of the crude virus material (that is, the virus in the plasma of infected chicks) made the program possible and, to a large extent, determined the course it followed and the interpretations that could be drawn. In view of their possible implications in other investigations, these will be discussed in more detail.

Of primary importance in an analysis of antigens is the quality of the antigenic material, a factor determined to a great extent by the source of the antigen. The ideal material can be described readily as a high concentration of pure antigen. When the antigen concerned is a virus, such a material can rarely be approached. However, it is the degree to which this first criterion is fulfilled that is of paramount importance. In the case of myeloblastosis, the presence of some 10^{12} virus particles per ml. in certain selected chicken plasmas provided an unusually acceptable source of virus antigen. Simple repeated cycles of ultracentrifugation and resuspension of the pellets in saline produced an antigenic material of great potentiality. Also important in the interpretation of the results was the fact that the virus was not exposed to drastic procedures such as tissue homogenization and to the debris of artificially ruptured cells. The virus particle studied was basically the same infectious agent that was found in the plasma of the infected chicken.

Sources of antigen of such a quality are not readily obtained, and perhaps are uniquely rare in the case of the tumor viruses. It should be pointed out that the use of plasma as a starting material has probably been neglected in the study of the etiology of many tumors. Another promising material, although perhaps a limited one, is milk.⁵ Tissue culture fluids are another potential source. The material of least promise, and the source most frequently used, is homogenized tumor tissue. Only in the case of the rabbit papilloma, with its very high concentration of virus in primarily keratinized tissue, are the properties of the tumor material favorable.¹

Another aspect of importance was the possibility of applying several different techniques to the measurement of virus concentration. It had previously been demonstrated that infectivity titers, particle counts, and ATPase activity were all quantitatively related to each other.⁷⁻¹⁵⁻¹⁷ This made feasible a flexibility in the choice of method or methods. Thus, for example, while the necessity for utilizing plasmas of high virus concentration was indicated above, the virus content of the plasmas from individual chickens is extremely variable. The ATPase method provided a means of screening chickens selectively by a rapid and simple procedure that made possible the use of all plasmas immediately after collection, and yet the plasmas were of uniformly high titer.¹¹⁻¹⁴ The variability in the virus content (only 5 per cent of the chickens with well-developed leukosis were satisfactory sources) is an obvious possibility to be recognized in other investigations.

The final point of emphasis is that all methods involved the use of techniques of demonstrated accuracy and precision. It was particularly necessary to develop the serologic and infectivity measurements to a level compatible with the physical techniques. As one example, in the determination of the sedimentation constants of the antigens, physical errors became important if more

than 90 per cent of the virus was removed. This made it necessary to utilize complement-fixation techniques that would yield quantitative values over a total concentration range of approximately tenfold.

The critical nature of these several aspects of the techniques and materials involved will be demonstrated many times in the discussion and interpretation of the results.

Analysis of the Antigenic Properties of the Virus

Four specific immune sera were utilized in reactions with the infected plasmas or virus concentrates. These were: (1) antiviral chicken immune serum prepared by immunization of young adult chickens with a series of doses of concentrated virus; (2) antiviral rabbit immune serum prepared with similar virus concentrates; (3) anti-chick-tissue immune serum produced in the rabbit by inoculation of homogenates of the liver and spleen of normal chickens; and (4) Forssman antibody obtained by immunization of the rabbit with guinea pig kidney.

All rabbit sera were heated at 56° C. for 30 min. and were absorbed with 4 changes of sheep red blood cells unless the Forssman antibody titer was to be measured. It should be emphasized that the names applied to these sera are derived only from the kind of material inoculated into the animal and do not necessarily reflect any implied specificity of the serum.

The reactions between these sera and the various antigens are summarized in TABLE 1. There were virtually complete cross reactions between all 4 immune sera and the 3 antigens—virus, chicken tissue, and Forssman antigen (sheep red blood cells).¹⁰ Of the many possible combinations, several tests were not attempted but, of those carried out, only the precipitation reactions between the several rabbit sera and the virus were negative. Since the precipitation of the virus by the antiviral chicken immune serum occurred only at the largest concentrations available, the negative results may presumably have been due to the inadequate amounts rather than to any specific properties of the reagents.

TABLE 1
REACTIONS OF THE IMMUNE SERA AND ANTIGENS

Immune serum	Antigens and tests				
	Virus			Chicken tissue	Sheep red blood cells
	Neutralization	Complement fixation	Precipitation	Complement fixation	Forssman lysis
Virus (rabbit).....	+	+	0	+	+
Virus (chicken).....	+	—	+	—	—
Chick tissue (rabbit).....	+	+	0	+	+
Forssman.....	+	+	0	—	+

Symbols: +, positive; 0, no measurable reaction; —, no test.

* Measured as inhibition of the Forssman antibody by the virus.

Some of the reactions presented in TABLE 1 require further amplification. Thus, while all 4 sera neutralized the virus, there were obvious differences between them. With the exception of the Forssman antibody, the sera neutralized the infectivity of the virus to the extent of at least 4 log intervals (the maximum amount that could be measured quantitatively). The highest concentration of a potent Forssman serum produced a reduction of only one log in residual virus titer. This was not the result of a simple difference in the effective titers of the sera, since the slope of the line relating the log decrease in virus titer to the amount of serum was much more shallow for the Forssman antibody.

A most significant point was that the virus and chick tissue immune sera produced in the rabbit were indistinguishable.¹⁰ Whether the immune sera were tested by neutralization of the virus or fixation of complement with either virus or chicken tissue as antigen, there were no qualitative or significant quantitative differences between the 2 types of immune sera. The sera produced in chickens were basically similar to these 2 classes of rabbit immune sera, but differed in that generally the titers were higher when tested by neutralization, and quantitative studies disclosed certain differences in the pattern of neutralization.

Homogeneity of the Virus Concentrates

The multiplicity of reactions of the virus preparations with the various immune sera raised the obvious possibility that the virus concentrates were mixtures of particles of different antigenic natures. Two hypothetical classes of mixtures can be visualized: (1) the material consists of the virus particles with certain other antigenic particles either much larger or much smaller than the virus or, (2) the technique of concentration in the ultracentrifuge selects a population of particles similar in size, but varying in their antigenic characteristics.

The possible presence of particles of chick tissue antigen of mass significantly different from that of the virus was evaluated in terms of the methods of ultracentrifugation and electron microscopy. When diluted filtered plasmas were used, the particulate material could be sedimented onto agar at high speed, and the constitution of the samples could be observed with the electron microscope. The photomicrographs demonstrated that, in addition to the virus particles, there were relatively fixed amounts of amorphous large particulate materials and some spheres smaller than the virus. The proportion of this material to the mass of virus could be reduced by the choice of plasmas containing high concentrations of the virus. It is important to note that repeated cycles of ultracentrifugation had little effect on the relative amount of these extraneous materials that may have been derived from the chicken tissues. For these reasons, both the experimental procedures and the production of immune sera were based on the use of plasmas containing only the highest concentrations of virus.

A second possible normal chicken antigen was a component of lower particle weight, in this case, plasma proteins. It would be expected that two components of such widely different particle weights as the virus and the plasma pro-

TABLE 2

DISTRIBUTION OF THE ATTRIBUTES OF THE VIRUS ON REPEATED
ULTRACENTRIFUGATION AND SUSPENSION IN SALINE

	Virus count per cent	Chick tissue titer	Forssman antigen titer
Plasma.....	100	—	—
First concentrate.....	74	63	56
Second concentrate.....	97	28	60
Third concentrate.....	68	24	57

teins could be separated by a series of successive ultracentrifugations and re-suspensions in saline. In a series of experiments, it was found that two cycles of ultracentrifugation and washing did, indeed, decrease the chick antigen titer, but only to a level at which it remained relatively constant on further treatment. One such example is shown in TABLE 2. The effect of ultracentrifugal concentration and washing on Forssman titer was even less, a result in accord with the observation that, under the conditions of measurement, normal chicken plasmas had little or no titer.

The conclusion to be drawn from these experiments was that a high level of chick-tissue and Forssman antigenicity remained associated with the dominant population of particles. These same particles had previously been demonstrated to possess the properties of infectivity and ATPase activity.

The other conceivable origin of heterogeneity was approached in a different manner. The method of preparative ultracentrifugation is basically a coarse method of isolation, most successfully applicable to mixtures of colloidal particles of very different sizes. It was possible that the virus concentrates consisted of mixtures of particles of nearly the same size, but of very different antigenic structure. Thus, some of these particles could be the virus and others the normal chicken particulates responsible for the chick-tissue and Forssman antigenicities. Separation of these two hypothetical populations of particles was attempted by two independent physical methods. One was a more discriminating method of ultracentrifugation by which rather subtle differences in sedimentation rates could be detected; the other was electrophoresis of virus concentrates, by means of which the particles could be separated on the basis of their electric charges in defined media.

The general concept was the same for both methods. Samples were drawn after the gravitational or electric field had been operating. The distribution of virus particles, ATPase, chick-antigen titer and Forssman titer was then measured. The calculated sedimentation constants were 722, 607, and 694 for the virus particle count, chick-tissue antigen, and Forssman antigen, respectively. In the electrophoresis experiments, using 4 samples at pH 7.0 and 2 at pH 8.5, the calculated mobilities for all of the measured characteristics including ATPase activity were within the experimental range of the value calculated on the basis of the visible boundary. The results of the application of two different fields of force, one based on the mass of the particles and the other on their electric charges, gave definite evidence that the chick-tissue and

Forssman antigens were integral parts of the infectious virus particles as found in the plasma of the chicken.¹⁰

The precipitation of virus in plasma and in concentrates by antiviral chicken immune serum gave direct evidence concerning the homogeneity of the virus of myeloblastosis.^{9, 10} Plasmas with maximal virus particle counts and immune serum were mixed and, after 1 to 3 min. of incubation, the samples were diluted and examined with the electron microscope. In all cases the results indicated that virtually all particles were aggregated by the immune chicken serum, and also that, aside from these aggregated particles, little other particulate material was present in the plasmas.

Thus, in summary, the application of combined serologic and physical techniques gave consistent evidence that the infectious agent, as found in chicken plasma, was composed of a rather uniform suspension of virus particles. These same particles contained an antigen or group of antigens that reacted with all three immune sera prepared with the various antigens—virus, chick tissue, and guinea pig kidney.

The Specificity of the Immune Sera

The analysis having reached this stage of exposition, the nature and specificity of the immune sera became questionable. Thus, if the virus possessed as many as three distinct antigens, the inoculation of rabbits with virus concentrates could produce an immune serum with a variety of specific capabilities. In addition to the production of obvious Forssman antibody, specific antibody to chick-tissue antigen would be expected. However, the possibility of antibodies to virus antigens could not be discounted. The latter could presumably be of importance in the neutralization reaction.

Considerable variation of the immunization procedure was attempted in an effort to improve the specificity of the serum at the source. In all procedures for the production of immune sera, emphasis was placed on the use of virus antigens of the highest available quality. The plasmas used were only those with the greatest virus content, and these were subjected to four cycles of ultracentrifugation and washing. This, as previously discussed, met the criterion of minimizing any extraneous antigens in the immunizing inocula.

However, if the several antigens were present on the virus particles, there still remained the possibility that antibodies specific for the virus might be disclosed, despite the dominance of the chick-tissue antigenicity. Modified adjuvants and courses of immunization were utilized, but no distinctive properties of the immune sera were altered. The use of smaller animals (mice and guinea pigs) was adopted so that relatively larger doses of virus could be used. These varied immune sera were tested for changes in their patterns of complement fixation when chick-tissue homogenate and concentrated virus were used as antigen. The results disclosed no consistent augmented or modified specificity of the sera.

Absorption of antiviral rabbit immune serum was performed in an attempt to disclose the presence of antibodies specific for the virus antigens. Forssman antibody was readily removed by absorption with several changes of sheep red blood cells, a procedure routinely applied to all immune sera produced in the

rabbit with chicken-tissue or virus inocula. Following this, the immune serum was exhaustively absorbed with several changes of homogenates of chicken tissues. When such absorbed sera were used in the complement-fixation test with chicken tissue as antigen, decreases of antibody titer up to 90 per cent were measured. However, with virus concentrates as antigen, the same tests disclosed a comparable decrease in titer. In addition, the capacity of such absorbed sera to neutralize the infectivity of the virus had been decreased by the same order of magnitude. Such results are in accord with the previously demonstrated ability of anti-chick-tissue immune rabbit sera to neutralize the virus as effectively as did the antiviral serums.

Antigenic Character of the Virus

The antigenic analysis of the virus of avian myeloblastic leukemia disclosed considerable evidence of the presence of a component of the host as an integral part of the virus particle. This was manifested in the presence of two separable types of antigens of probable host origin: (1) the Forssman antigen and (2) an antigen indistinguishable from that of chicken tissue. The evidence included both the constant association of such host antigens with the virus particles and the neutralization of the virus by antibody to host tissue.

The origin of such a component of the virus is of interest. It should be remembered that the source of the virus was the plasma of the chicken, and that virtually no laboratory manipulation was required to obtain virus for many of these experiments. The colloidal physical state of the virus makes the absorption of host components feasible. However, there are only two places where such antigens could be absorbed, either in the plasma of the chicken or in the infected cell. The plasma is a poor source of such antigens; the Forssman titers of normal plasmas are negligible compared to those of the virus, and the pattern of complement fixation by chicken tissue is clearly distinguishable from that of plasma. Unless the serologic characters of these two antigens are modified by absorption to the virus, the plasma does not appear to be the source of the host component of the virus. A possibly related property of the virus, ATPase activity, similarly is found only in negligible amounts in the normal plasma. At present, the evidence indicates that the host antigens and enzymatic activity are properties of the virus as it leaves the cell.

Whatever the origin of the host component, it must be emphasized that these antigens are an integral part of the infective virus particle. As such the antigenic properties of such chicken components would not be without influence on the process of infection. In certain respects, infection with such a virus might share certain of the restrictions imposed on the transplantation of tumors. While the evidence for the presence of host antigens as integral parts of the virus is well documented, it is questionable whether any specific viral antigen has been demonstrated. The presence of antibody to such an antigen in rabbit immune serum has no experimental basis. The immune mechanism of the rabbit responds to the virus exclusively as if the material consisted of chicken tissue. The development of antibodies to the virus in the chicken is the only evidence of a specific viral antigen. Reconciliation of such contrary results is not indicated in the experimental results. It is conceivable that the

dominance of chicken tissue hinders the demonstration of any specific viral antibodies in rabbit serum by the methods available. Only when this effect is partially nullified by the use of homologous animals are such antibodies readily demonstrable. Another possible explanation would hinge on the degree of specificity of the antibody production. Could the same antigenic material be handled basically as chicken antigen by the rabbit, while at the same time the antibody-forming mechanism of the chicken would respond to it as a foreign antigen?

The results arrived at in this investigation should not be considered as applicable to other viruses associated with tumor formation.^{11, 12} The demonstration of host antigen as a part of a virus is limited to one other case, namely, the influenza virus.¹³ The application of these results resides more in the definition of certain criteria that must be fulfilled before precise antigenic characterization of the tumor viruses can be achieved.

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IMMUNOLOGICAL RELATIONSHIPS BETWEEN VIRUS AND CELL IN THE ROUS SARCOMA*

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Over the past twenty-five years, a series of contradictory reports has appeared from various laboratories concerning the antigenic relationship between normal chicken tissues and the Rous sarcoma virus. One school of workers (Gye and Purdy, 1931; Amies and Carr, 1939) has reported that antiserum to normal chicken tissues neutralizes the infectivity of the virus, while a second school (Keogh, 1938; Kabat and Furth, 1941; Barrett, 1940) has failed to detect neutralization with such antisera. Subsequently, Dmochowski (1948) reported the absence of cross reaction on complement fixation between Rous sarcoma virus and normal tissues. A clearly positive specific neutralization would have great significance, since it would suggest that an unaltered host component forms an integral and functional part of the virus surface.

The reduction in virus titer upon treatment with an antiserum to normal cells, as reported by the earlier workers, could be due to any one of a number of mechanisms other than the direct and specific neutralization of the virus particle. Such collateral effects fall into two classes: (1) an effect of the anticell serum on the cells of the animal host used in assay due to inoculation of the undiluted serum-virus mixture; and (2) a nonspecific action on the virus particles, such as clumping or coprecipitation, due to inability to free the virus of contaminating normal tissue components.

The first class of effects can be divided further into several categories. The anticell serum might suppress the multiplication of cells infected with a tumor virus, and thus give a low estimate of the number of infective particles in a given preparation. That an anticell serum, even when made in a homologous species, can suppress tumor-cell multiplication in the whole animal has been demonstrated unequivocally by Gorer and Amos (1956). These workers showed that antiserum to mouse leukemia cells can inhibit tumor formation completely in the mouse, even when given one day after implantation of the leukemia cells.

The other possible mechanisms for spurious reduction in virus titer through action of an anticell serum on the cells are based on findings with nontumor viruses. Quersin-Thiry (1955) has reported that anticell sera, by their action on the cell, can interfere with virus adsorption (presumably by covering the cell receptors), multiplication, or release of a number of necrotizing viruses. There is no a priori reason for thinking that tumor viruses should not be affected in a like manner, and all these effects must therefore be taken into consideration. Some of these effects might require inoculating the serum at the same site as the virus; others are effective even if inoculated at a distant site.

There are also examples in the literature to illustrate the second class of

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collateral effects. Hardy and Horsfall (1948) have shown that an irreversible union frequently occurs between influenza virus and a normal mucoprotein found in allantoic fluid and in cells. It is therefore not surprising that Knight (1946) found that influenza virus could be precipitated with antiserum to normal tissues. However, this clumped virus retained its infectivity when diluted and assayed by the end-point technique. It is apparent that if the serum remains through the inoculation of the mixture into the host animal, thus maintaining the virus in clumps, there might be an apparent drop in titer due to the fact that a clump would be greatly restricted in the number of cells that it can infect, compared to the case in which its constituent particles could infect cells independently. However, clumping requires collisions between virus particles, and it is therefore highly dependent upon virus concentration. Calculations from the Smoluchowski formula (Smoluchowski, 1917) show that the collision frequency in one hour for particles about 100 m μ in diameter is negligible below a concentration of 10^6 particles per ml. Therefore, all the following experiments were carried out with minimal virus concentrations.

The above considerations make it imperative to establish some criteria for specific virus neutralization. These can be provided empirically by careful comparison of the effects due to the anticell serum with those due to neutralization by an antiviral serum obtained from a convalescent animal. Fortunately, a specific serum against the Rous sarcoma virus was supplied by W. Ray Bryan of the National Cancer Institute, Bethesda, Md. The present study constitutes first a confirmation of the apparent neutralization of the Rous sarcoma virus by antichick serum, and second, a demonstration through comparison with antiviral serum that apparent neutralization by antichick serum is not due to a direct neutralization of the virus particle, but to action of the antibody on the cells. The results suggested that the discrepancies in previous reports were due to the different techniques used in the various laboratories to carry out the neutralization tests (Rubin, 1956).

Materials and Methods

The materials and methods have been described in earlier papers (Rubin, 1955, 1956). In brief, the virus was assayed by counting tumors produced on the chorioallantoic membrane (CAM) of the developing chick embryo. Antiserum to normal chicken tissues was made by repeated inoculation of homogenized chick-embryo suspensions into rabbits. An antiviral serum was obtained through the courtesy of W. R. Bryan from a chicken that had recovered from infection.

Experimental Confirmation of Apparent Neutralization

The purpose of the first experiment was to find whether there was any evidence of neutralization by antichick serum under optimal conditions for combined serum action both in the tube and on the CAM. Undiluted serum was incubated with virus and complement for one hour at 4° C., and the mixture placed on the CAM. The number of tumors resulting was compared with a control in which normal rabbit serum was substituted for the antichick serum.

TABLE 1

APPARENT NEUTRALIZATION OF ROUS SARCOMA VIRUS BY ANTISERUM TO NORMAL TISSUES,
AND REVERSAL OF EFFECT BY DILUTING BEFORE INOCULATION

Virus incubated with	Dilution of mixture before inoculation	Relative infectivity
Normal serum 1:5	None	1.00
Antichick serum 1:5	None	0.087
Antichick serum 1:5	1:100	1.13
Antiviral serum 1:50	None	0.033
Antiviral serum 1:50	1:100	0.13
Antiviral serum 1:5000	None	0.94

The results may be seen in the first two rows of TABLE 1. The antichick serum reduced the number of tumors by more than 90 per cent.

Reversibility of Apparent Neutralization

Recent work with a variety of animal viruses (Dulbecco, Vogt, and Strickland, 1956, with poliomyelitis and Western equine encephalomyelitis; Rubin and Franklin, 1957, with Newcastle disease virus) has shown that most neutralized virus particles cannot be reactivated by simple dilution of the serum virus mixture. It was of interest to determine whether this held true with antiserum to the Rous sarcoma virus. If this were true, and if the antichick serum also acted directly on the virus in a similar manner, one would expect a large component of the virus population to be neutralized irreversibly in the latter case as well. On the other hand, if the apparent neutralization of Rous sarcoma virus by antichick serum was indirect and due to an effect on the host cells, then diluting the serum virus mixtures before inoculation would be expected to abolish the effect.

Various mixtures of the antisera and virus were made, always using a great excess of antiserum. The virus was used at concentrations that would give a countable number of tumors after the appropriate dilution when plated on the CAM. Some of the mixtures were plated directly; others were diluted before plating (see Rubin, 1956, for details). The results are presented in TABLE 1. It is clear that the effect produced with antichick serum is abolished if the mixture is diluted before inoculation, while the inactivation by antiviral serum that is of the same order of magnitude is at least 90 per cent irreversible by simple dilution.

This finding indicates that the reductions in virus titer with antiviral and antichick sera are due to different mechanisms, and that the latter acts chiefly after the mixture has been inoculated into the host.

The Requirement for Complement

Some previous reports have emphasized the requirement for complement in order to obtain apparent neutralization with antichick serum (Gye and Purdy, 1931). On the other hand, although complement may enhance neutralization of some viruses by specific antiviral serum (Leymaster and Ward, 1949), it is not required for such neutralization (Dulbecco, Vogt, and Strick-

TABLE 2
THE ROLE OF COMPLEMENT IN APPARENT NEUTRALIZATION

Virus incubated with	Relative infectivity
Normal serum + complement.....	1.00
Heated antichick serum.....	0.56
Heated antichick serum + complement.....	0.032
Heated antiviral serum.....	0.019
Heated antiviral serum.....	0.015

land, 1956). The role of complement in bacterial and red blood cell lysis is universally recognized, and recent work has shown that it is required for animal cell lysis (Kalfayan and Kidd, 1953; Morgan, 1955; Schrek and Preston, 1956). A strong requirement for complement by the antichick serum would therefore be consistent with the hypothesis that apparent neutralization is mediated through action of the antichick serum on the host cells. To investigate this requirement, heated antichick and antiviral sera were tested for their capacity to reduce the apparent virus titer in the presence and in the absence of complement. The results in TABLE 2 show that only the antichick serum required complement in order to exert its titer-reducing effect.

The Addition of Serum After Infection

Recent work in this laboratory has shown that a virus particle adsorbed to a susceptible host cell rapidly penetrates that cell at 37° C. and then can no longer be inactivated by antiviral serum. This observation was utilized in the following experiment to determine conclusively whether antichick serum acted on the virus or on the cells. Thirty-six eggs were infected with equal amounts of Rous sarcoma virus. One group of 6 was left without further treatment, while other groups received either antichick or antiviral serum at appropriate intervals after infection. The results are presented in TABLE 3. They show that the antiviral serum has little effect if added 1 hour after the virus, while all effect was gone at 4 hours. However, the antichick serum retained its full effectiveness if added as late as 2 days after infection, showing that it acted on the cells, since action on the virus was no longer possible. It is likely that the anticell serum must be inoculated at the same site as the virus, since it is so quickly adsorbed by the cells.

TABLE 3
THE EFFECT OF ADDING SERUM AFTER INFECTION

Hours after infection at which serum was added	Normal rabbit serum	Relative infectivity after adding	
		antichick serum	antiviral serum
1	1.00	0.028	0.56
4	—	0.096	1.09
48	—	0.064	—

TABLE 4
THE EFFECT OF ANTICKICK SERUM ON VACCINIA ASSAY ON
THE CHORIOALLANTOIC MEMBRANE

Vaccinia plus	Relative infectivity
Normal serum.....	1.00
Antickick serum.....	0.75

Effect of Antickick Serum on Vaccinia Virus Titration

The foregoing experiment shows that the antickick serum acts to reduce the apparent virus titer after the first stages of infection, but it does not distinguish between suppression of the cells' multiplication and suppression of their ability to produce virus.

To help discriminate between these alternative mechanisms, the effect of antickick serum was tested on the titration of a necrotizing virus, vaccinia, which produces its lesion chiefly through infection and reinfection of cells, thus causing a pock of necrotic cells. The results, which may be seen in TABLE 4, show that the antickick serum has no significant influence on the titration of vaccinia virus, indicating that the cells retained the capacity to produce virus. This suggests that cells infected with the Rous sarcoma virus may also continue to release virus after treatment with the antickick serum. This leaves as the most likely explanation for apparent neutralization the hypothesis that the infected cells treated with antickick serum can no longer multiply to form a tumor.

The Effect of Forssman Antibody on Titration of the Rous Sarcoma Virus and Adsorption of Antibodies

Chicken cells are known to contain Forssman antigen; therefore, an investigation was made of the possibility that Forssman antibody might simulate the action of antickick serum in suppressing tumors. An antiserum to boiled sheep red blood cell stroma was kindly supplied by José Vinas of Johns Hopkins University, Baltimore, Md., and was substituted for antickick serum in the neutralization procedure. The results in TABLE 5 show that such heterogenetic serum is almost as effective as antickick serum in suppressing tumors of the CAM. It could also be shown that some of the tumor-suppressing activity of the antickick serum could be removed by adsorption with sheep red blood

TABLE 5
EFFECT OF ANTISERUM TO BOILED SHEEP RED BLOOD CELL STROMA
ON THE ASSAY OF ROUS SARCOMA VIRUS

Virus incubated with	Relative infectivity
Normal serum.....	1.00
Antickick serum.....	0.01
Antisheep red blood count.....	0.04

TABLE 6
 ADSORPTION OF SERA WITH CHICK EMBRYO TISSUES OR WITH
 SHEEP RED BLOOD CELLS

Virus incubated with	Serum previously adsorbed with	Relative infectivity
Normal serum	—	1.00
Antichick serum	—	0.044
Antichick serum	Chick tissues	0.86
Antichick serum	Sheep red blood cells	0.37
Antiviral serum	—	0.13
Antiviral serum	Chick tissues	0.13

cells, and most of it could be removed by adsorption with chicken cells (Rubin 1956). Adsorption with chicken cells had no effect on the antiviral serum (TABLE 6).

Discussion

The results presented here counsel caution in interpreting results of virus neutralization with anticell serum. In the last few years detailed analysis of neutralization by specific antiviral sera, using the most advanced techniques for manipulation and assay, has revealed unique characteristics that might serve as criteria for evaluating the effects of anticell sera on the apparent infectivity of virus preparations. Among these characteristics of specific neutralization are the largely irreversible nature of the virus-antibody union under physiological conditions; the requirement for only a single antibody molecule at a viral site to cause inactivation as revealed in exponential inactivation kinetics, and a linear dependence of inactivation rate on antibody concentrations; the failure of antiviral antibody to exert any effect upon the virus once it has penetrated the cell; the lack of requirement for complement; the absorption of antibody only with specific viral material (Dulbecco, Vogt, and Strickland, 1956; Rubin and Franklin, 1957; Rubin, in press). When some of these criteria were tested with the Rous sarcoma virus, they were found to hold only for the action of convalescent antiviral sera, and they failed in every case for antichick sera. Consequently, it was concluded that there is no compelling evidence that normal host components form an integral part of the virus structure.

The results presented here have recently been confirmed by T. Borsos of Johns Hopkins University, Baltimore, Md. (personal communication). This investigator has made the further important observation that when the Rous sarcoma virus is assayed by a technique that does not involve cell proliferation (the hemorrhagic lesion technique in the chick embryo) there is no effect of antichick serum. This provides strong support for the idea that antichick serum reduces virus titer, in the case of the CAM titration, by suppressing cell multiplication.

Also relevant is the report of Habel *et al.* (1957) that anticell sera can inhibit the assay of a variety of viruses in tissue culture by acting on the cells. These

authors stress the danger of drawing false conclusions about the possible antigenic relationships of all viruses due to the presence of cellular material in the samples used for immunization.

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MODIFICATION OF THE IMMUNE RESPONSE BY RADIATION AND CORTISONE

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In considering the modification of the immune response by ionizing radiations or by cortisone, I shall largely limit this review to the antibody response following a single injection of a nonreproducing antigen. This limitation will avoid many of the complexities that arise during long-continued immunization and infection. Representative difficulties that this procedure enables us to avoid involve the proper assessment of recovery, the variable antigen dosage due to the continual release of antigen during infection, and the proper differentiation of the role of connective tissue cells and antibody in phagocytic immunity. I shall try to relate the various observed effects to antibody synthesis. The relevant data for such a correlation are drawn mostly from recent work on the differential sensitivity of the various parts of the antibody response in the rabbit to X rays and to cortisone and on various protective measures that have been found to neutralize one or more of the suppressive manifestations of these agents. Such an attempt necessitates a critical review of the literature in some respects as contrasted to extrapolations from meager data in other respects.

Radiosensitivity of Different Stages in the Primary Immune Response

In general, the earlier publications agree that antibody formation is more intensely suppressed when antigen is given a short time after severe total-body X radiation rather than before it (Benjamin and Sluka, 1908; and reviews by W. H. and L. G. Taliaferro, 1951, and Talmage, 1955). Recent work has substantiated and extended these results. By injecting antigen at closely spaced intervals before and after severe total-body irradiation, it has been found that there is a very short period at the first part of the immune response in the rabbit to bovine gamma-globulin (Dixon, Talmage, and Maurer, 1952) and to Forssman antigen of sheep red cells (Taliaferro, Taliaferro, and Janssen, 1952), which determines whether or not antibody formation is markedly suppressed. When this period is completed before X-ray damage (that is, when antigen is given shortly before irradiation), the remainder of the immune process is *relatively* radioresistant in that normal amounts of antibody are formed. The simplicity of dividing the immune response into a radiosensitive early event and a radioresistant antibody-producing stage, as pictured by Dixon, Talmage, and Maurer, has led some recent investigators to overlook the fact that all parts of the immune response are probably affected by X radiation, although to markedly different extents.

The differential effects of X rays on the timing and amount of antibody production may be illustrated by the results obtained on the hemolysin response in the rabbit to the Forssman antigen of sheep red cells (Taliaferro, Taliaferro, and Janssen, 1952, and W. H. and L. G. Taliaferro, 1954b). Parenthetically,

TABLE 1*

THE EFFECTS OF TOTAL-BODY IRRADIATION (500 R) ON HEMOLYSIN PRODUCTION^{††} AS INDICATED BY PEAK TITER AND THE TIME AND AVERAGE RATE (k_1) OF FORMATION

Group	Time of red cell injection	Mean peak titer		Average rate (k_1) of accumulation	Length in days of:		Number of rabbits
		Log	Arithmetic $\times 10^3$		Induction period	Production period to peak titer	
Red cells before 500 r							
1	4 days	2.76 \pm .35	1.31	0.4 \pm 0.04	4.1 \pm 0.6	9.3 \pm 2.6	6
2	2 days	3.07 \pm .31	2.60	0.4 \pm 0.11	7.2 \pm 1.6	9.5 \pm 2.3	5
3	2 hours	3.74 \pm .14	8.08	0.6 \pm 0.05	7.5 \pm 0.7	10.5 \pm 0.8	10
Red cells after 500 r							
4	1 hour	3.29 \pm .16	2.94	0.8 \pm 0.14	7.7 \pm 0.5	7.2 \pm 1.4	6
5	4 hours	2.63 \pm .14	0.67	0.7 \pm 0.11	9.3 \pm 0.3	7.8 \pm 1.5	8
6 $\frac{1}{2}$	1 day	1.49 \pm .10	0.04	0.3	8.4	7.6	1(9)
7	1 week	2.54 \pm .26	0.59	0.4 \pm 0.09	8.8 \pm 2.1	11.4 \pm 2.5	4
8	3 weeks	2.75 \pm .42	1.57	0.9 \pm 0.38	5.7 \pm 0.4	6.0 \pm 1.3	5
9	8 weeks	3.41 \pm .26	7.10	1.0 \pm 0.20	3.7 \pm 0.6	4.9 \pm 0.7	12
Red cells alone: no X rays							
10	Controls	3.25 \pm .09	2.82	1.0 \pm 0.08	3.6 \pm 0.19	5.2 \pm 0.47	31

* From W. H. and L. G. Taliaferro (1954b).

[†] All values are means \pm standard errors. Data from individual rabbits were obtained from fitted curves, such as those shown in FIGURE 1. The titers in this table and in the figures are expressed in terms of 50 per cent hemolytic units/ml. serum (see especially W. H. and L. G. Taliaferro, 1950 and 1956). For statistical analyses, log peak titers are used because of the nature of the antibody curves and because log values are normally distributed, whereas arithmetic titers are markedly skewed. Other parameters such as the length of various periods and rate constants are treated arithmetically.

[‡] Only one rabbit gave a response in Group 6. In this group, mean peak titer was calculated by considering the peak titer of the anergic rabbits as equal to the preimmunization titer; the remaining constants are for the single animal that gave an antibody response.

it should be stated that the rise and fall of this antibody has been shown to follow a discontinuous curve, each segment of which is described by the well-known "compound-interest" integral. Peak titer has been found to be the best single relative measure of the amount of antibody formed, whereas the length of the induction period indicates the time of appearance of antibody production, and the average rate constant (k_1) of antibody rise to peak titer indicates the relative rate of antibody production (TABLE 1).

In nonirradiated rabbits, after one intravenous injection of a small amount of sheep red cells, an arithmetic peak titer of about 2800 50 per cent hemolytic units/ml. serum occurs after an induction period of 4 days and an initial rise of 5 days at an average k_1 rate of about 1.0 (TABLE 1, Group 10). The injection of antigen at various times *before* or *after* intense total-body irradiation modifies the normal response. The response in 4 irradiated groups will be described briefly.

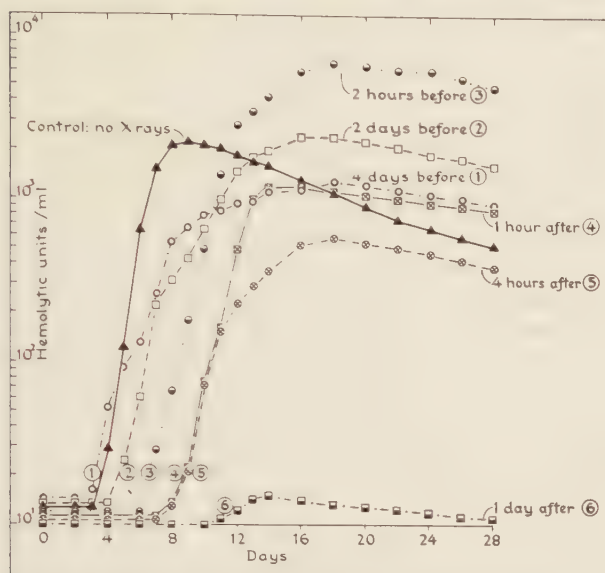


FIGURE 1. Mean hemolysin curves from 6 groups of rabbits for 28 days, following 1 intravenous injection of sheep red cells (0.125 ml. of 1 per cent/kg.) given from 4 days before to 1 day after total-body irradiation with 500 r. A control mean hemolysin curve from nonirradiated rabbits is included. The time of injection of antigen in relation to X rays is given at the right of each curve (from the data of W. H. and L. G. Taliaferro, 1954b). Means for peak titer and other measurements of hemolysin formation are given in TABLE 1.

Note that the induction period is normal, and almost normal amounts of antibody are produced, although at a slower rate, when antigen is given 4 days before X rays (Curve 1), and that all aspects of hemolysin production either are decreased or retarded when antigen is given 1 day after X rays (Curve 6). Intermediate effects are produced in Curves 2 through 5, with abnormally large amounts of hemolysin produced in Curve 3.

(1) Maximal X-ray injury is evident when antigen is injected about 12 hr. to 1 day after severe total-body irradiation. At this time peak titer is drastically reduced and, aside from a large proportion of the rabbits in which no antibody is formed, the induction period and length of the initial rise are each radically increased and the average k_1 rate is decreased (TABLE 1, Group 6).

(2) When antigen is injected very shortly *after* the time of irradiation before injury to the antibody-forming mechanism has been completed, nearly normal amounts of antibody are produced, but during a prolonged period of rise at a decreased rate (TABLE 1, Group 4).

(3) This same delay followed by a slower rate of antibody formation leading to normal or even above normal amounts of antibody occurs when antigen is injected several hours *before* irradiation (TABLE 1, Group 3).

(4) When antigen is injected 4 days *before* irradiation, antibody reaches a peak titer within the normal range although at a slower rate (TABLE 1, Group 1). In this group, X rays do not affect the induction period because they are given after the induction period has been completed.

Mean peak titers for these 4 groups of rabbits as well as additional ones are shown in TABLE 1 and FIGURE 1.

Differences from the foregoing X-ray effects on antibody formation have been reported in other species, but there is no conclusive evidence that they are fundamental in character. Since there may be less difference between the radiosensitivity of different parts of the immune response, however, most emphasis will be placed on work on the rabbit. Results with the other species will be briefly reviewed here.

Kohn (1951) found that the injection of sheep red cells into the rat before the administration of 175 r, 350 r, or 600 r results in a delayed and lower peak titer and a slowing of the rate of hemolysin decline after peak titer. These effects were more pronounced when antigen was given after irradiation, although complete suppression was not obtained. Another group of workers, however, found that the hemolysin response is completely suppressed in the rat when red cells are injected 1 or 6 days after administering 500 r, and is markedly suppressed when antigen is injected at various intervals before irradiation (Wissler *et al.*, 1953, and Fitch *et al.*, 1956). Taken together, these results indicate that the early part of the immune response in both the rat and the rabbit is radiosensitive, but that the later part (the period of antibody production) is more radiosensitive in the rat. Part of this difference can be accounted for by the fact that the half life of the rat hemolysin is approximately 0.8 days (data from Kohn, 1951), whereas the half life of the rabbit hemolysin is 2.8 days (Taliaferro and Talmage, 1956).

Mice injected with alum-precipitated tetanus toxoid 6 days after administration of 450 r gave no evidence of protective antibodies when challenged with 10 minimal lethal doses (MLD) of tetanus toxin 14 days later. Almost identical results were obtained when the toxoid was given 3 days before irradiation (Silverman and Chin, 1955). Later work indicates that the apparent inhibition of antibody formation is a delay and not a reduction in the total amount of antibody formed (Silverman and Chin, 1956). This situation is similar to the findings for the hemolysin response in the rabbit, except that the mouse shows a greater radiosensitivity of the later part of the immune response.

In further characterizing the radiosensitivity of the immune response, I shall try to link it directly with antibody synthesis. All recent work indicates that antibody synthesis involves the antigen-induced synthesis of a new serum globulin directly from amino acids and not the modification of pre-existing serum globulin (Gros, Coursaget, and Macheboeuf, 1952, and Green and Anker, 1954).

We are just beginning to obtain evidence on the relation of the induction period and the rise and fall of serum antibody to the actual incorporation of amino acids into antibody. Thus, when a donor rabbit is given S^{35} -labeled amino acids during the induction period of a secondary response to bovine serum albumin, and its spleen is removed, minced, washed, and injected into a nonlabeled recipient, the antibody formed in the recipient is labeled only to a very small degree, that is, not over 1 per cent and probably about 0.3 per cent (Taliaferro and Talmage, 1955). This result indicates that the antibody formed in the recipient is formed from the recipient's amino acids and, conversely, that there are no long-lived amino acid-containing precursors formed

during the induction period that later appear in the antibody. Dixon *et al.* (1956) reached similar conclusions for the primary response of the rabbit to bovine gamma-globulin, but suggest that synthesis may increase logarithmically, beginning at a very low level during the induction period of the secondary response. The final result, the possible synthesis of a very small amount during the first three days, is not essentially different from our results. Previously, Green and Anker (1954), using several isotopically labeled amino acids in a study of the secondary response of rabbits to ovalbumin, reached the conclusion that 31 per cent of the amino acids in the antibody on the fifth day were withdrawn from the amino acid pool during the induction period. I believe their results arose from the recycling of labeled amino acids and from the inclusion of labeled coprecipitating antibody and labeled complement in the antigen-antibody precipitate. These errors were obviated in our work by transferring the antibody-synthesizing mechanism to an isotope-free environment. Taken as a whole, these results strongly suggest that the induction period is one during which the antibody-synthesizing mechanism (special cells, enzymes, or other mechanisms) are formed.

The antibody-synthesizing mechanism, in line with work on protein synthesis in microorganisms and mammals, once present, quickly assembles the amino acids from the amino acid pool into antibody. The S^{35} label appears in antibody within 40 min. during active antibody synthesis in the rabbit (W. H. and L. G. Taliaferro, 1957b). This length of time is remarkably close to the interval between the administration of C^{14} -labeled glycine and the appearance of radioactivity in serum proteins of the rabbit (Green and Anker, 1955) and in pancreatic proteins of the rat (Junqueira, Hirsch, and Rothschild, 1955). After the intravenous injection of antigen into an animal in which the spleen is particularly active in antibody formation and in which there is no evidence of tissue storage, the foregoing results suggest that the rise in serum antibody gives a fairly accurate estimate of actual synthesis.

In analyzing the immune response further with respect to X-ray damage, I shall discuss the following three parts that have been differentiated by antibody titrations, amino acid incorporation, and X-ray effects: (1) the very short preinduction period during which some necessary event takes place to initiate the rest of the process; (2) the induction period proper or the period in which the antibody-synthesizing mechanism is developed; and (3) the period of the rise of serum antibody or of antibody synthesis.

Preinduction period. This period is highly radiosensitive, as is most clearly shown in the rabbit. Interest in the events occurring in this period derives from the fact that an understanding of their nature may explain the internal milieu necessary to allow the body to initiate the production of antibody-synthesizing mechanisms.

Both Dixon, Talmage, and Maurer (1952) and Taliaferro, Taliaferro, and Janssen (1952) attempted to ascertain the length of the preinduction period. This determination is probably impossible to measure because the power to form antibody drops so rapidly that there is no satisfactory base line for the calculations. Thus, as shown in TABLE 1 and FIGURE 2, rabbits injected with sheep red cells 1 hr. after irradiation have a mean log peak hemolysin titer

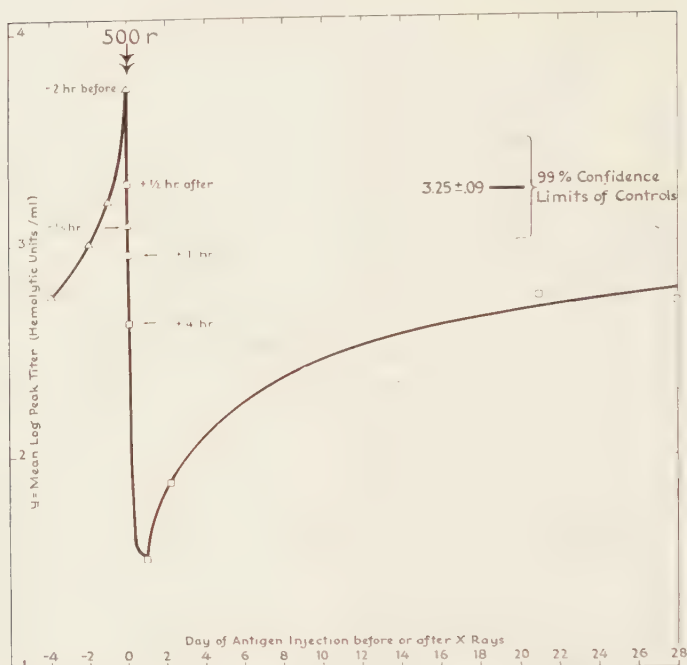


FIGURE 2. Mean log peak hemolysin titer from 14 groups of rabbits following 1 intravenous injection of sheep red cells (0.125 ml. of 1 per cent/kg.) given from 4 days before to 4 weeks after total-body irradiation with 500 r (from the data of W. H. and L. G. Taliaferro, 1954b).

Note the rapid decline in hemolysin production (as measured by peak titer) when antigen is given from 2 hr. before to 4 hr. after X rays, and the gradual recovery when antigen is given from 1 day to 4 weeks after X rays.

not significantly different from that of nonirradiated controls, whereas those injected 4 hr. after irradiation form significantly less antibody, but considerably more than those injected 1 day later. From such data, we can conclude only that the processes in this period may be almost instantaneous and do not take over 3 hr. to reduce mean log peak titer by log 0.63 or to approximately 22 per cent of the normal response (W. H. and L. G. Taliaferro, 1954b).

The dosage response curve in FIGURE 3, when antigen is injected after 700 r, indicates that there is a fairly sharply defined threshold for the X-ray inhibition of hemolysin production (W. H. and L. G. Taliaferro, 1954a). Thus, 100 r results in no depression of mean log peak titer, whereas 400 r suppresses it about 95 per cent. The median suppressive dose is approximately 240 r (see also the work of Silverman and Chin, 1955, on antitoxin production in the mouse).

The recovery of hemolysin peak titer from irradiation damage is logarithmic. Visual inspection of a graph of log peak titer versus log time from 1 day to 8 weeks indicates that 50 per cent recovery of log peak titer takes about 10 days. At this time, however, the arithmetical peak titer has reached only 13 per cent of the normal value. On the other hand, 50 per cent recovery can be calculated

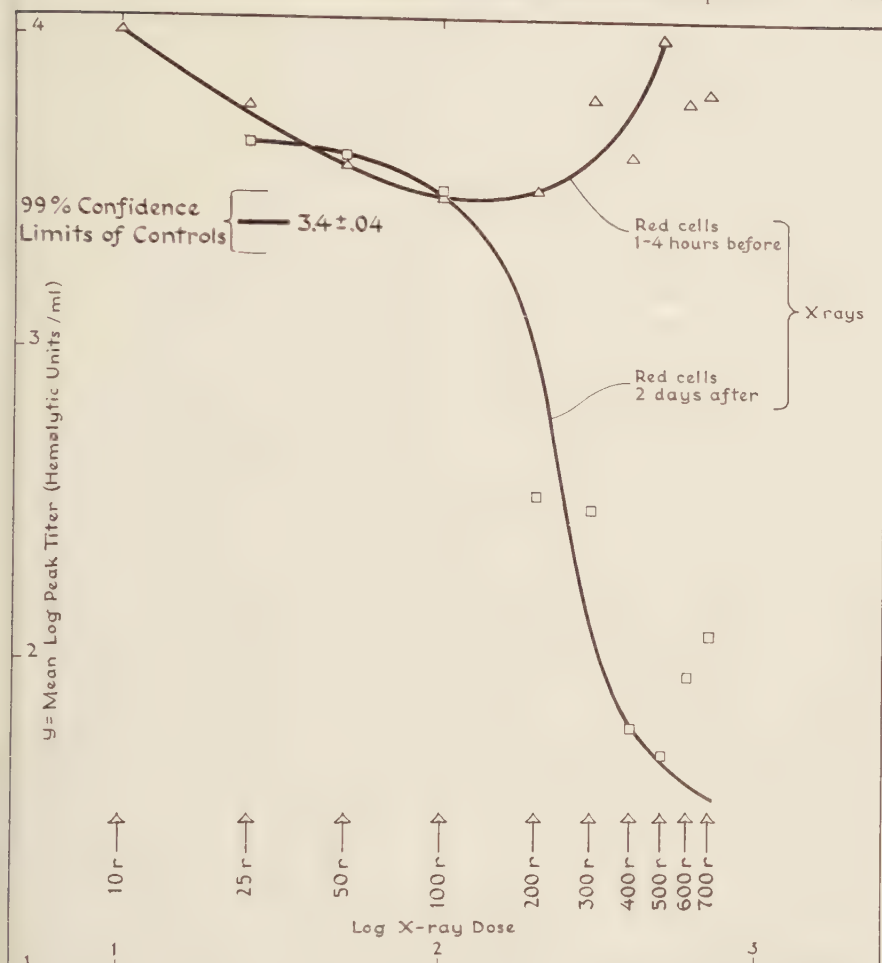


FIGURE 3. Two dosage-response curves relating mean log peak hemolysin titer to log dose of X rays (1) from 10 groups of rabbits following 1 intravenous injection of sheep red cells (1.25 ml. of 1 per cent/kg.) given 1 to 4 hr. before total-body irradiation with 10 r to 700 r, and (2) from 9 groups of rabbits injected with the same amount of antigen 2 days after administration of the same amounts of X rays (from the data of W. H. and L. G. Taliaferro, 1954a).

Note the enhancement of antibody production (as measured by peak titer and as compared to the controls) in Curve 1 at 10 r and at 500 r and the sharp decline in antibody production in Curve 2 from 200 r to 400 r.

as 4.05 ± 1.6 days by a probit analysis in which suppression is considered to be a log peak titer of 2.2 50 per cent units (a value outside the normal range) or less. Complete recovery occurs by 8 weeks (TABLE 1, Series 9, and W. H. and L. G. Taliaferro, 1954b).

It has been suggested that the preinduction period may be related to the initial events involved in the fixation of antigen by the cells (Kohn, 1951), to the localization of the antigen in the antibody-forming sites or to the early

metabolism of the antigen (Taliaferro, Taliaferro, and Janssen, 1952), or to the persisting adaptation of the gamma-globulin-synthesizing mechanism to the specific antigen (Dixon, Talmage, and Maurer, 1952). From the cellular standpoint, it has been suggested that irradiation damages a precursor of the antibody-forming cells or prevents certain cells from reaching a critical stage at which they can react properly with the antigen (Talmage, 1955). So far, no marked difference has been found in the gross localization of antigen following severe total-body X irradiation (Fitch *et al.*, 1953, and Ingraham, 1955), but this finding does not necessarily contradict the ideas of highly specific types of localization implied in the above suggestions. Furthermore, particulate antigens, when localized in macrophages, may be largely digested and thus prevented from stimulating antibody formation.

It is a reasonable working hypothesis that sufficient radiation completely stops the production of some extracellular or intracellular material necessary for the completion of the early radiosensitive event during the preinduction period (Dixon, Talmage, and Bukantz, 1951). The results shown in FIGURE 2 indicate that this material has a biological half life of about 2 hr. for hemolysin production. About twice the half life has been found for the formation of antibody against bovine gamma globulin (Dixon, Talmage, and Maurer, 1952).

Data on the factors necessary for the preinduction stage have been obtained at the cellular and noncellular levels by studies on recovery or protection from X-ray damage. In considering this work, it should be pointed out that restoration of the antibody-forming capacity in a rabbit made anergic by irradiation involves the radiosensitive preinduction period, since completion of this stage is necessary to attain normal titers. Thus, in rabbits X-rayed after this stage has been completed, normal or even above-normal titers can be reached (Series 1 through 4 in TABLE 1).

It was first found that rabbits previously X-rayed with 525 r form antibody against dysentery bacilli when they are injected with washed lymph-node cells previously incubated with bacteria or their soluble antigens or when the cells and bacteria are injected separately (Harris and Harris, 1955, and Harris, Harris, and Farber, 1954 and 1955). Somewhat similarly, a mince or extract of normal rabbit spleen, when mixed with red cells and injected into rabbits previously X-rayed with 400 r (\approx ED₉₅), gives partial but significant restoration of the antibody-forming capacity as measured by peak titer (Jaroslow and Taliaferro, 1956). The restorative principle in spleen is nonspecific and probably not necessarily associated with living cells. Thus, extracts of normal mouse spleen are as effective as those of normal rabbit spleen. In addition, suspensions or extracts of HeLa cells (a tissue culture strain of human cervical carcinoma) as well as a simple yeast autolysate are the most effective (FIGURE 4). Minces of normal rabbit kidney or muscle are ineffective. Preliminary experiments with the yeast autolysate indicate that the restorative principle is only partially inactivated or lost by heating at 90° C. for 10 min., by dialysis against tap water for 15 hr., or by a combination of both treatments. Unpublished experiments indicate that the effective principle is a product of the action of the specific nuclease on ribonucleic or deoxyribonucleic acid.

Lead-shielding the surgically exteriorized spleen or appendix during other-

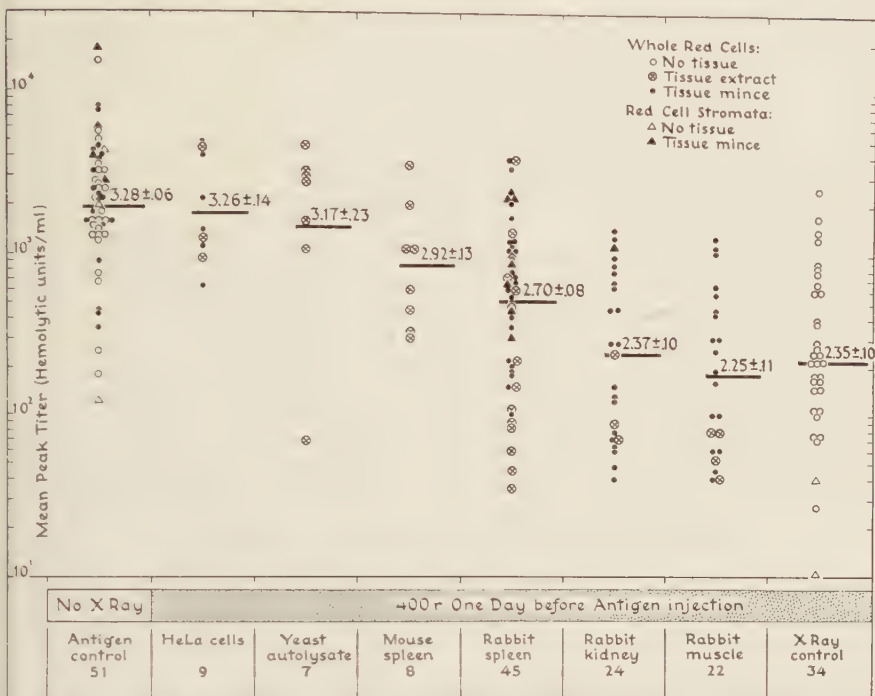


FIGURE 4. Log peak hemolysin titer in 6 groups of rabbits following 1 intravenous injection of a mixture of antigen (1 ml. of 10 per cent sheep red cells/kg. or 2×10^8 heated sheep red cell stromata/kg. and one of 6 preparations 1 day after total-body irradiation with 400 r. Two control groups are included. The mean for each group is shown by a bar (adapted from Jaroslow and Taliaferro, 1956).

Note that the suppression of peak titer due to X rays is completely overcome by preparations of HeLa cells and yeast autolysate, but not by preparations of kidney or muscle. Mouse and rabbit spleen preparations partially protect against X ray suppression of peak titer.

wise total-body irradiation of the rabbit protects the capacity to form red cell hemolysin (Jacobson, Robson, and Marks, 1950, and Jacobson and Robson, 1952), but the protection is highly variable in amount and at times nil due to factors that are not well understood (W. H. and L. G. Taliaferro, 1956). Both Jacobson and Robson (1952) and Wissler *et al.* (1953) relate the protection afforded by the lead-shielded spleen to the ability of the spleen to form antibody. This association is probably fortuitous. Thus, shielding diverse organs or sites such as the spleen, 75 per cent of the liver, or the hind legs results in approximately 18 per cent protection of antibody production, whereas shielding the appendix results in complete protection (Süssdorf and Draper, 1956). In such experiments when a single injection of antigen is given intravenously, the spleen forms a major part of the antibody, whereas the appendix forms little, if any. It seems logical to assume that the efficacy of shielding depends upon the protection of particular components in the shielded tissue that cause the regeneration of injured antibody-forming tissue *in situ* or elsewhere. The mechanism of regeneration may result from mobile cells and/or acellular ma-

terial (see Loutit, 1954, and Jacobson, 1955 and 1956) akin to the material in yeast autolysate that restores antibody formation in previously irradiated rabbits (Jaroslow and Taliaferro, 1956).

Easy generalizations no longer can be made on the function of a given cell in antibody formation based on an apparent parallel radiosensitivity of a particular cell and antibody formation. Thus, lymphocytes, which are markedly radiosensitive (Bloom, 1948), have often been associated with antibody production (review in W. H. and L. G. Taliaferro, 1951). Rabbits injected with antigen 4 days before irradiation, however, usually form normal amounts of hemolysin during the time when most of the lymphocytes are destroyed, that is, for 9 days after irradiation (TABLE 1, Group 1). Recently, more specific correlations have been attempted.

Fitch *et al.* (1956) picture the histological sequence of hemolysin production in the rat as beginning with the uptake of antigen by reticular cells, the proliferation and transformation of these cells into large basophilic or pyroninophilic cells, variously classified as lymphocytes and young plasma cells (Taliaferro, 1949) and, eventually, into plasma cells. Fitch and his associates believe that radiation has little effect on fixation of antigen by the cell or upon antigen within or outside of it, but that radiation can inhibit antibody formation by inhibiting the proliferation of the antibody-forming cells or by inhibiting protein synthesis within them.

In a study of the radiosensitive secondary response to tetanus toxoid in the mouse, Spiers (1957) reported that the response of mononuclear cells is inhibited whether the antigen is given prior to or after irradiation, whereas the local concentration of eosinophils is markedly inhibited only when antigen is injected 1 to 14 days after irradiation and not when it is injected 24 to 72 hr. beforehand. Thus, the inhibition of eosinophil concentration by X rays parallels the radiosensitive preinduction period. Spiers believes that antibody production depends not only upon the differentiation and maturation of mononuclear cells, but also upon the migration of mature eosinophils into the area of antigen fixation where they take part in some early process in antibody formation.

Present experimental evidence does not preclude any of the possibilities that all stages of the antibody-forming process occur in the same cell, that the stages are divided among a series of generations from the same cell, or that the process is divided among different cells. Furthermore, X radiation may act by destroying key cells, or it may act without killing them by inhibiting their mitotic division or certain internal metabolic processes. That the last may occur is indicated by work on the X-ray inhibition of intracellular digestion in peritoneal phagocytes (Donaldson *et al.*, 1956).

Induction period. When severe total-body irradiation is administered after the preinduction period is completed, but before a major portion of the induction period has been completed, the appearance of antibody in the serum is markedly retarded, but the amount of antibody is not reduced and actually may be above normal in amount. In fact, the induction period is doubled in length even when X rays are given after half the normal induction period has been completed (TABLE 1, Group 2).

The lengthening of the induction period may be an expression of a retardation in the formation of the antibody-synthesizing apparatus. In any case, it probably involves mechanism(s) different from that operating during the preinduction period. This idea is suggested by experiments in which the two periods are affected independently. Thus, the induction period is lengthened, but normal or above-normal peak titers are eventually attained in rabbits given antigen a few hours before irradiation (FIGURE 1, Curve 2). A somewhat similar sequence occurs in irradiated rabbits given extracts of HeLa cells or yeast autolysate. Contrariwise, the induction period is nearly normal in length, but peak titer is greatly suppressed when the spleen is lead-shielded (while the rest of the rabbit is irradiated with 500 r 2 days before the injection of sheep red cells) and then removed 1 to 5 days after irradiation (W. H. and L. G. Taliaferro, 1956).

Long induction periods sometimes occur in animals injected with antigen after irradiation. When they extend beyond the point of considerable recovery (three or more weeks after irradiation), it is possible that the original reaction is more or less completely inhibited, and that antigen stored in the body initiates a response after sufficient recovery. This explanation may account for some of the long delays reported for antitoxin formation in X-rayed mice (Silverman and Chin, 1954 and 1955). It may also be related to the secondary rises at shorter intervals, as found in rats (Fitch *et al.*, 1956).

Production period or rise of serum antibody. The production period refers to the time during which antibody increases in the serum to peak titer and during which it decreases from peak titer when the rate of decline is less than the rate of metabolic decay for the antibody. It is probably the least radiosensitive of the three periods in the rabbit and cannot be modified to a discernible degree unless antibody is being formed at a rapid rate. In rabbits given 500 r at the beginning of the initial rise, hemolysin peak titer is practically normal, but the rate of rise is decreased (TABLE 1, Group 1). If our earlier analysis of the relation of the synthesis of antibody to the stages of the immune process is correct, this effect is a true reduction in the rate of synthesis that is induced while synthesis is in progress. As pointed out before, this period is apparently more radiosensitive in the rat and mouse than in the rabbit.

When yeast autolysate and HeLa cells counteract X-ray injury during the preinduction period, serum antibody also rises in the treated rabbits at a normal rate, but the induction period remains lengthened. It is not evident at present how such materials can influence the preinduction period and the rate of antibody rise without affecting the intervening induction period.

X-Ray Enhancement of Antibody Formation

Small amounts of X rays, often administered locally, have frequently been reported to hasten recovery from infections in man and experimental animals and to enhance antibody formation in experimental animals (reviewed in W. H. and L. G. Taliaferro, 1951). Many of these experiments have not been well controlled.

Among the most interesting studies are those that relate the increase in

antibodies to a hormonal mechanism associated with the pituitary-adrenal cortical control of the physiological activities of the lymphocyte (Dougherty and White, 1946 and 1947, and review by White, 1949). Unfortunately, many of these experimental findings have not been corroborated by other investigators (Taliaferro, 1949).

In our work, 3 experimental conditions have elicited a statistically significant enhancement of mean log peak hemolysin titer. These are: (1) when antigen is injected shortly before total-body irradiation of 300 r to 700 r, but not of 200 r (right side of the top curve in FIGURE 3); (2) when antigen is injected shortly before total-body irradiation of 10 r and 25 r, but not of 50 r (left side of top curve in FIGURE 3); and (3) when antigen is injected 2 days after the administration of 2000 r to 10,000 r, but not of 1400 r or less, to the surgically exteriorized spleen alone (FIGURE 5). In the first case, true stimulation does not occur because peak titer is reached only after a longer induction period and a slower antibody rise as compared to nonirradiated controls (TABLE 1, Group 3; and FIGURE 1, Curve 3). In the second case, the results, although

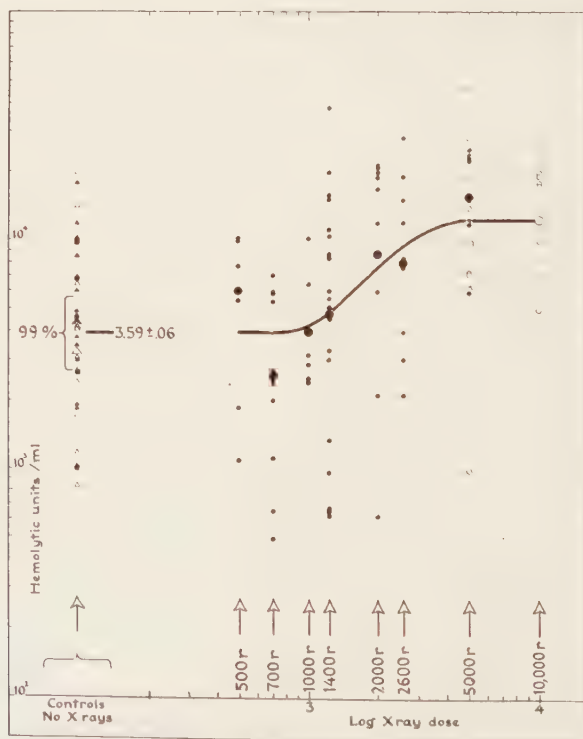


FIGURE 5. Dosage-response curve relating log peak hemolysin titer to log dose of X rays administered to the exteriorized spleen alone in rabbits receiving one intravenous injection of sheep red cells (1.25 ml. of 1 per cent kg. 2 days after irradiation). Log peak titers are shown for individual rabbits, together with the mean log peak titer (large data point) for each group. The solid and open circles represent different radiation factors (from W. H. and L. G. Taliaferro, 1956).

Note that above-normal amounts of hemolysin are produced with 2000 r or more.

statistically significant, may have been caused by extraneous factors, since variable results were obtained with different groups of rabbits. In the third case, true stimulation occurs, as evidenced by increased titers reached in normal or slightly less than normal times at higher rates.

Related to our results with severe radiation just after antigen injection may be the finding that 1000 r administered to the local site of a previous antigen injection and again one week later stimulates the production of hemolysins in the rabbit (Graham *et al.*, 1956). Stimulation, however, occurred only when the antigen was in the site at the time of the first irradiation. Somewhat similarly, 1000 r at the site of the injection of alum-precipitated toxoid enhances circulating diphtheria antitoxin and especially local antitoxin (Graham and Leskowitz, 1956).

Little is known regarding the mechanisms involved in the enhancement of antibody formation by X radiation. Various earlier suggestions have been reviewed (W. H. and L. G. Taliaferro, 1951). Graham *et al.* suggest a direct stimulation of the antibody-producing cells by ionizing radiation. We (1952) earlier pointed out that the enhancement simulates the type of metabolic stimulation sometimes observed with low concentrations of cellular poisons or at the beginning of the action of high concentrations of such poisons. At present, we believe the mechanism is related to that involved in the restoration of the antibody-forming capacity in previously irradiated rabbits by such factors as yeast autolysate and spleen mince. This idea originally was suggested by the fact that the precipitous fall in the power to form antibody in the rabbit is linear from the time of antigen injection 1 to 4 hr. before to 4 hr. after irradiation with 500 r (FIGURE 2). As a working hypothesis, we believe that a considerable amount of some material necessary for antibody formation is released from certain cells damaged by radiation. This material quickly disappears from the irradiated animal and reappears later as a part of the recovery process. It is, moreover, instrumental in the formation of increased amounts of antibody if present in sufficient amounts at a critical time.

X-Ray Effects on the Anamnestic Response

Some secondary responses have been found to be relatively radioresistant (the response to bovine gamma globulin, Dixon, Talmage, and Maurer, 1952, and to crystalline egg albumin administered with Freund's adjuvant to the rabbit, Silverman and Chin, 1954), whereas others have been found to be radiosensitive (the hemolysin response in the rabbit, Taliaferro, Taliaferro, and Janssen, 1952, and the response to tetanus toxoid in the mouse, Hale and Stoner, 1952 and 1954). Various suggestions have been made to explain the radiosensitivity of the secondary hemolysin response, but I know of only 3 experimental studies relating to it. As a result of his work, Ingraham (1955) believed that it is due to the less effective antigenic stimulation by red cells that are rapidly removed from the blood stream, as contrasted to the long-continued stimulation of bovine gamma globulin, which remains in the blood stream for several days. In support of this view, sulfanilazo bovine globulin leaves the blood stream rapidly, as contrasted to bovine gamma globulin. Furthermore, the secondary response to this azo antigen, as tested by haptenic

hemolysis, becomes radiosensitive in contrast to the radioresistance of the secondary response to bovine gamma globulin (Hill, 1954). Unfortunately, in this work, the antibody responses were very low and very few animals were studied.

Talmage, Freter, and Thomson (1956) found that the amount of red cells used for the initial immunization and for the anamnestic response is an important factor in determining whether the secondary hemolysin response is radiosensitive or not. After intense multiple initial immunization and the injection of a large amount of red cells to elicit the secondary response, radiosensitivity of the anamnestic response markedly decreases after 500 r. These investigators believe that the intense initial immunization causes the immune system to be more responsive to a second injection of antigen, and that large amounts of antigen are needed to initiate a response after irradiation, because the injured immune mechanism is less efficient and the antigen is functionally blocked by circulating antibody already present. The correctness of the first suggestion is shown clearly in a study of the regression of log peak hemolysin titer on log amount of antigen over a two-thousandfold range from 0.125 to 250 ml. of 1 per cent sheep cells per kg. rabbit (W. H. and L. G. Tالياferro, 1954a). Within this range, for each amount used, the antibody response is less in previously irradiated than in normal rabbits. The regression coefficient, however, is almost 3 times higher in the X-rayed than in the normal animals; that is, a given increase in amount of antigen is relatively 3 times as effective in increasing the antibody response in X-rayed rabbits. The data are shown in FIGURE 6. Therefore, should the same linear relationship hold for higher amounts of antigen, an amount could theoretically be given to previously irradiated and normal rabbits that would induce identical antibody responses. Actually, such an extrapolation would require the impossible amount of about 3×10^3 ml. of 1 per cent cells. The second suggestion is based on the finding that appreciable amounts of circulating antibody prevent small amounts of injected red cells from stimulating the antibody-forming mechanism (Talmage, Freter, and Thomson, 1956).

Crosland-Taylor (1955) made the interesting observation that an apparent resistance of the secondary response may be due to the fact that the break in the ability to form antibody, as shown in FIGURE 2, takes place later with tetanus toxoid than it does in the primary hemolysin response shown in that figure. Thus, the antibody-forming capacity is not decreased significantly, as compared to the controls, when antigen to induce a secondary response is injected 6 hr. or 2 days after irradiation, but is decreased by 1.22 log, that is, to one sixteenth, when injected 10 days after irradiation. If the foregoing conclusion proves generally applicable the radioresistance of a secondary response is essentially the extension of the period after irradiation during which the injection of antigen will result in the formation of normal amounts of antibody after a delay. Moreover, if our previous assumption is correct that the sharp decrease in antibody-forming power is due to the decay of some material necessary for the initiation of antibody formation, less of the material may be needed to produce antibody during the secondary response, and inhibition may thereby be delayed.

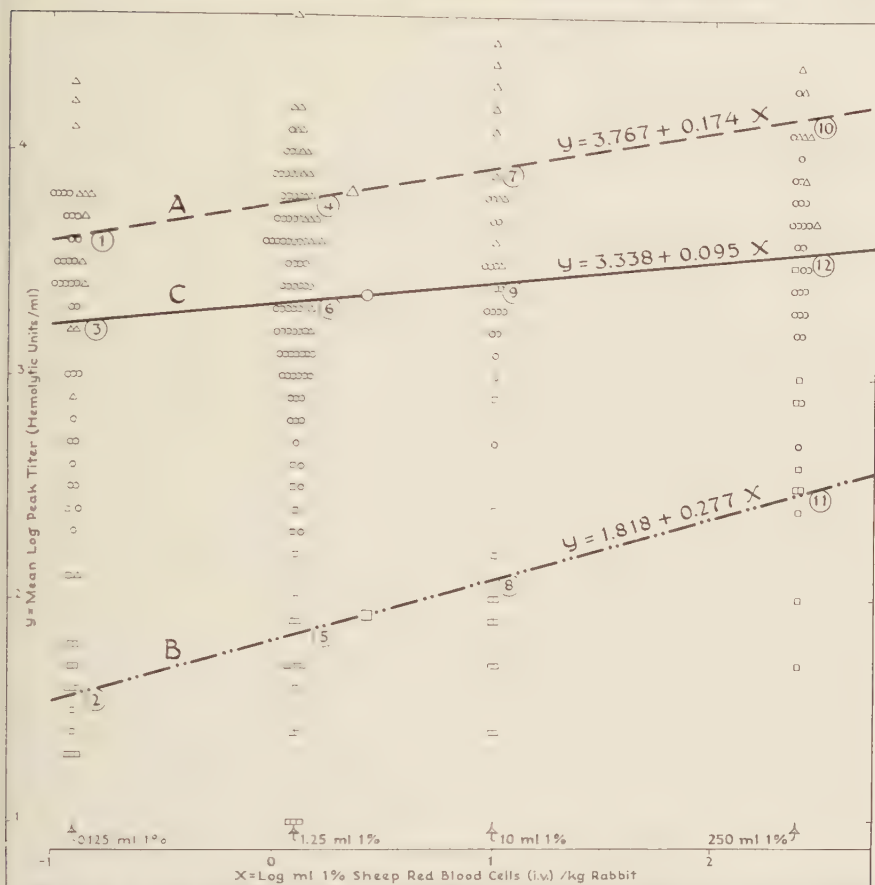


FIGURE 6. Regression of the log peak hemolysis titer on log antigen dose (A) in 4 groups of rabbits receiving 1 intravenous injection of 4 different amounts of sheep red cells (from 0.125 to 250 ml. of 1 per cent/kg.) given 1 to 4 hr. before total-body irradiation with 500 r or 600 r and (B) in 4 groups of rabbits receiving the same amounts of antigen given 2 days after the same amount of irradiation. (C) Non-irradiated groups are included. Titers for A, B, and C are indicated by triangles, squares, and circles, respectively (from W. H. and L. G. Taliaferro, 1954a).

Note the enhancement of peak titer in A and the suppression in B throughout the whole antigen dose range as compared to C. Also note the higher regression coefficient in the X-rayed animals, especially in those receiving antigen after irradiation (B).

Should this delay last until regeneration takes place, the response would be apparently radioresistant. From these ideas, it follows that all secondary responses may be relatively radioresistant because of the increased power of the previously immunized animal to respond to antigen, but may be radio-sensitive under certain conditions, that is, when too little antigen is used or the initial immunization is insufficient to sensitize the immune mechanism properly.

*Cortisone Suppression of Antibody Formation During the
Primary and Anamnestic Response*

There seems little question that large amounts of cortisone and other adrenal steroids, given repeatedly, suppress antibody formation. Excellent reviews of the subject have been made by Fischel (1953) and by Kass and Finland (1953). Experimental details and results are well illustrated in the careful studies with cortisone in the rabbit by Bjørneboe, Fischel, and Stoerk (1951), Germuth, Oyama, and Offinger (1951), and Malkiel and Hargis (1952).

The results may be illustrated by a set of experiments by Bjørneboe, Fischel, and Stoerk (1951). Rabbits were given 10 mg. of cortisone acetate daily beginning 2 days before and extending throughout 4 weeks of immunization. Immunization consisted of an initial intravenous injection of 2×10^9 formalin-killed pneumococci followed by twice that number at approximately 2-day intervals. Under such conditions, agglutinins measured quantitatively are markedly reduced as compared to controls receiving no cortisone. The reduction is less marked, but significant in amount, when the same dosage of cortisone is started on the fourteenth day of immunization after antibody production is well established. The smaller effect during the production phase of antibody formation is similar to the results with X radiation, as reported by W. H. and L. G. Taliaferro (1957a).

Germuth, Offinger, and Oyama (1952) found that cortisone suppresses antibody formation in the guinea pig, but they believe that this species is more resistant to the effect of the steroid than is the rabbit.

The recent work of Berglund and Fagraeus (1956) on rats does much to bring the results with cortisone in line with those on the X-ray inhibition of antibody formation. Thus, the effects of cortisone obtained during the hemolysin response in rats following a single injection of sheep red cells are dependent on hormone dosage, antigen dosage, and the time of administering the antigen (Berglund and Fagraeus, 1953). Maximum antibody suppression occurs when 3 doses of cortisone (4 mg. 100 gm. rat day) are given beginning 2 days before and ending on the day of antigen injection. Cortisone in similar amounts, given after the injection of antigen, is ineffective. The apparent absence of lesser effects when cortisone is given later may be accounted for by the fact that titrations could not be spaced sufficiently closely to determine rates of rise. Considered together, these findings indicate the presence of an initial cortisone-sensitive stage in antibody formation (Berglund, 1956a). Increasing the amount of antigen in a single dose decreases the effectiveness of the hormonal suppression of hemolysin formation (Berglund, 1956b) just as it does in X-ray suppression (W. H. and L. G. Taliaferro, 1954a).

X-ray treatment was divided somewhat similarly and a single injection of a formalized preparation of H antigen of *Salmonella paratyphi* B given by Clemmesen and Andersen (1948). In 3 groups of 3 rabbits, each animal received 5 irradiations of 120 r (total 600 r) spaced at 3-day intervals. Under such conditions, antibody formation is significantly suppressed when antigen is injected after the 5 irradiations or between the second and third irradiation, but is not suppressed when antigen is given before the series of irradiations or

after only 2 doses of 120 r. The results of the first and third series are in line with recently published work by W. H. and L. G. Taliaferro (1957a).

Hemolysin production can also be restored in the cortisone-treated rat with spleen or thymus cells (Berglund and Fagraeus, 1956). One intraperitoneal injection of cells is effective when given separately on the day of an intraperitoneal antigen injection. In contrast to the results reported for X rays (Jaroslow and Taliaferro, 1956), grinding the cells in a homogenizer destroys the restorative action. This difference may be accounted for by differences in the experimental conditions, such as the degree of antibody suppression and the amount of antigen.

Cortisone also markedly suppresses the anamnestic response in the rabbit to crystalline ovalbumin (Fischel, Vaughan, and Photopoulos, 1952). This finding is of particular interest because the secondary response to this antigen is resistant to 400 r in the rabbit (see discussion above on the radiosensitivity of the secondary response, and Silverman and Chin, 1954).

Cortisone has no effect on the biological half life of homologous antibody passively transferred to the rabbit (Fischel, Stoerk, and Bjørneboe, 1951). This result is in line with X-ray data (Hollingsworth, 1950, and unpublished work by the author) and indicates that a suppression of serum antibody actually represents a decrease in the rate of antibody synthesis.

Among the various ideas regarding the mechanism of antibody suppression by cortisone, Kass and Finland (1953) suggest that a common mechanism may be involved in the suppressive action of the steroid on antibody synthesis, the local inflammatory response, and the ability of macrophages to break down and dispose of foreign materials. As stated earlier, however, it is uncertain at present whether phagocytosis and digestion of antigen by macrophages are a necessary prelude to antibody formation, or whether these processes remove and thereby prevent a major portion of the injected material from exerting its antigenic capacity.

Striking similarities have been described in the histological changes produced in lymphoid and myeloid tissues by various so-called lymphocytocidal agents, such as ionizing radiations, the nitrogen and sulfur mustards, and cortisone. Present data indicate that identical or similar mechanisms may be involved in the modification of the antibody response by X radiation and cortisone. Further work may well extend this parallelism of action to the other lymphocytocidal agents.

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DISCUSSION: PART III

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RENATO DULBECCO: A word should be said about the considerable part that the immunological phenomena observed in the field of viruses have played in this section of this monograph. The significance of these phenomena is twofold. On the one hand, viruses are the recognized agents of some cancers and are regarded as the hypothetical agents of others. Consequently, knowledge of the immunological phenomena of viruses has a direct bearing on the etiology of cancer and, perhaps, on its ultimate control. On the other hand, immunological analysis can be pushed further in the study of viruses than in the study of any other biological objects. In fact, viruses behave like any other cellular proteins in precipitation and complement-fixation studies. In addition, viruses have a highly specific function; namely, the prevention of this function by antibody, termed neutralization, affords another avenue of investigation of immunological phenomena. In turn, the viral function can be divided into many steps, such as the attachment of the virus to the cell, its penetration into the cell, and its multiplication in a series of cycles. Each of these steps may be separately affected by antibody, and these separate effects may be detected in appropriate experiments. Thus, the study of the neutralization of viruses may afford the finest possible analysis of an immunological phenomenon and of the interplay of the antibody action with the biological function of the material that is exposed to antibody.

In this approach, however, we must be very careful. As Horsfall pointed out earlier in this monograph, a student of neutralization may encounter important pitfalls. These dangers seem all the more threatening when the study of neutralization is carried out by the method analyzed extensively in Horsfall's paper, the method in which neutralization is studied by bringing both the virus and the antibody into contact with the susceptible cell. Actually, we have learned from Horsfall's presentation that this kind of approach will yield nothing but pitfalls.

The situation is better when it is possible to study neutralization without exposing the cells to the antibody. Consequently, it appears that, in immunological work with viruses, it is essential that a proper method be used, and that the analysis be of a quantitative nature as far as possible.

These and other points have been developed extensively by the contributors to this publication.

ISIDOR GREENWALD (*University of Buffalo School of Medicine, Buffalo, N. Y.*): I should like to ask Witebsky whether his technique permits the differentiation between the thyroglobulin of normal and diseased thyroids. There certainly must be different varieties.

ERNEST WITEBSKY (*University of Buffalo School of Medicine, Buffalo, N. Y.*): We have tried to compare extracts of human goiter with the extracts of normal thyroid tissue. So far we have been unable to detect any difference. In fact,

the goiter extract proved to be more effective than the normal thyroid extract, presumably because of its high content of thyroglobulin.

In order to distinguish possible differences that might occur between these two types of thyroglobulin, we applied the technique of inhibition of agglutination. In this way we have thus far been able to prove the specificity of the thyroid extract as such in contrast to that of the other organ extracts. The latter extracts did not inhibit agglutination at all.

PIERRE GRABAR (*Pasteur Institute, Paris, France*): When we discussed the different immunological techniques, the hemagglutination technique was not mentioned. In my own paper I commented upon the pitfalls of Boyden's technique; with standard cells it works beautifully and is probably the most sensitive method when only one antigen is present, but when you have a mixture of antigens one cannot know which of the antigens is actually fixed on the tanned cells. Therefore, one has great difficulty, for example, in adsorbing the serum with the noninteresting antigens. I observe that you have merely touched upon this cross reaction with human serum. Some of the organ extracts react with several sera prepared in the same manner, but as each individual animal produces different antibodies against different constituents of a mixture, by this agglutination technique one sometimes finds cross reaction, but in other cases one does not, and that is due to the fact that the tanned cells do not fix regularly all the antigen present in the mixture.

ERNEST WITEBSKY: I quite agree with Grabar's comment regarding the difficulties presented by the tanned-cell agglutination test. It took us about two years to master this test, but once the technique is learned it really works beautifully.

ROBERT A. GOOD (*Medical School, University of Minnesota, Minneapolis, Minn.*): One wonders whether the experimental disease that is produced by the injection of thyroid tissue along with the adjuvants is due to the antibody that has been demonstrated and reported in these studies. I should like to ask Witebsky whether it has been possible to transfer the experimental thyroiditis by the administration of serum, whether passive transfer can be achieved with the cells of a diseased animal, or whether both cells and serum are needed.

ERNEST WITEBSKY: I am frankly amazed that the changes brought about in the thyroid gland roughly parallel the titer of the circulating antibody. Possibly, in addition to the circulating antibodies, there are others that have not been discerned as yet. Thus far, we have had unsatisfactory results with passive transfer in our laboratory.

ISRAEL DAVIDSOHN (*Mount Sinai Hospital, Chicago, Ill.*): These results may throw some light on a problem that has been puzzling pathologists for a long time. Pathologists studying the human thyroid have seen granulomas not infrequently. I am not speaking of Hashimoto's disease or lesions of that type. Assertions have been made in the literature that foreign body giant cells are produced by colloid getting outside of the acini. Witebsky's results would support this contention, especially if it were possible to demonstrate autoantibodies in cases of this kind.

ERNEST WITEBSKY: As Davidsohn has observed, it is assumed in those instances in which thyroid antibodies are produced that the thyroglobulin is

released from the gland without being acted upon by the enzymes that usually catabolize them. The nature of the trigger mechanism that releases this thyroglobulin intact is still a mystery of the disease. It is assumed that the thyroglobulin gets into the circulation without being changed antigenically.

ROBERT A. GOOD: With reference to some of the points raised in the paper of Billingham and Brent, in our laboratory we have observed what we interpret to be evidence of this graft-versus-host reaction in the agammaglobulinemic patients. The agammaglobulinemic patients to whom I refer are those whom we have shown to be unable to form circulating antibody in response to antigenic stimulation and who have extremely low gamma-globulin concentrations as measured by immunochemical methods. We have been able to achieve successful homotransplantation of skin in such patients. In several of them we have also carried out homotransplantation of lymph nodes, using several different methods of implanting the lymph node tissue into a recipient agammaglobulinemic patient. Indeed, we were able to induce antibody formation. However, somewhat to our surprise, the antibody-producing capacity lasted for a period of only $2\frac{1}{2}$ months in one instance and for 2 months in another. In each of these cases the antibody formation that had been induced in the recipient patient ceased, and the nodes were destroyed.

There are several possible interpretations of this observation, one of which is the graft-versus-host reaction suggested here by Brent and Billingham. As a consequence of our own observations, as well as those of Brent and his colleagues, we have abandoned plans for attempting to introduce large amounts of lymphatic or hematopoietic tissues in agammaglobulinemic patients in an effort to restore their immunological capacity. Consequently, I would certainly agree with Brent and Billingham in expressing caution concerning future attempts to introduce perhaps larger amounts of lymphoid tissue into patients of this sort.

To return to the two agammaglobulinemic patients, the lymphoid tissue that was implanted in them certainly functioned for a time. These patients, who previously were unresponsive, showed immunological capacity as defined by the ability to form antibody against antigens to typhoid, paratyphoid, mumps, and poliomyelitis. This immunological capacity had not been present in the agammaglobulinemic patients prior to the transplantation. However, after a transient period of immunological responsiveness, immunological capacity was lost, and the nodes were destroyed. Christopher Martin has observed exactly the same thing in a patient with acquired agammaglobulinemia in whom he transplanted 8 lymph nodes. In this case, function persisted approximately 160 days, and then the nodes were destroyed both functionally and anatomically.

In addition to the hypothesis presented here, several other possible explanations for the failure of permanent takes of lymph node homotransplantations have been considered. For example, it seems possible that the temporary function of the transplant was merely a matter of inadequate primary transplantation of the lymphoid tissue on a technical level. To our view the fact that the lymph nodes did function as long as $2\frac{1}{2}$ months in our study and 160 days in Martin's study is strong evidence that a successful initial transplanta-

tion was achieved. The other possibility is that the agammaglobulinemic patients possess a limited ability to reject the homotransplanted tissue and can reject tissue from the functional point of view prior to rejection from the anatomical point of view. Thus, it is conceivable that one should interpret the skin transplantation observations in the agammaglobulinemic patients with caution and as evidence of decreased reactivity to homotransplants, but not its absence.

There is, of course, the question as to why a patient who was unable to produce gamma globulin was immune to a lymph node. The view I favor at the moment is that the graft is reacting to the host rather than the host to the graft. This interpretation would be in line with Brent and Billingham's interpretation of the deaths occurring following the intravenous introduction of spleen cells during the period of immunological immaturity in newborn mice.

MERRILL W. CHASE (*The Rockefeller Institute for Medical Research, New York, N. Y.*): An experience with less cogency than that reported by Good might be of some general interest here. In transferring lymph node cells from one set of guinea pigs to another set (all animals being produced within the same colony, but not having genetic homozygosity), J. R. Battisto and I have encountered a phenomenon that we have called "lymph-node disease." In a period of 2 to 5 days after the injection of the harvested cells into the recipient individuals, the latter sometimes show definite evidence of adenopathy; in these individuals petechial hemorrhages of the skin commonly develop until death may finally ensue, generally between the seventh and twentieth days. This phenomenon is not one in which we have found regression or atrophy within the lymph glands, but in these adult animals we have observed a process that may bear some analogy to that reported by Brent and Billingham.

ROBERT W. WISSLER (*Department of Pathology, University of Chicago, Chicago, Ill.*): I should like to refer to an experiment that is being performed by Joanne Denko and Eric Simmons at the Argonne Cancer Research Hospital of the University of Chicago. These investigators have been attempting to discover why irradiated animals that have been given spleen transplants die much earlier than would be expected if they had not been irradiated, even though their survival time is several months. This type of experiment has not been reported in detail, up to this time, for the excellent reason that the reactions that are present in the bone marrow, spleen, and lymph nodes are so remarkably variable that it is very difficult to find a common pattern to explain the deaths of the animals. At present, all that I can say is that, in studying these tissues with Denko and Simmons, I have been impressed by the highly histopathological appearance of these lymph nodes, spleens, and bone marrows. While one cannot predict the pattern in a given animal, some of them show severe suppression of the blood-forming capacity and a remarkable conversion to "blastic" cell types, usually of a single cell line.

LESLIE BRENT (*University College, London, England*): We have certainly observed a remarkable irregularity in the severity of the disease brought about by the reaction of adult spleen cells against the tissues of their tolerant hosts. In extreme cases the death of the hosts may ensue; such animals have been shown to be almost completely devoid of normal lymphoid tissue. However, the ma-

majority of tolerant mice (of the strain combination that was our principal research tool) were normal, to outward appearance, and were the victims of less dramatic damage. Furthermore, mice that could be shown to be only incompletely tolerant (that is, animals that sooner or later reacted against their grafts) displayed only very slight abnormalities of this kind. As would be expected, neither did their spleens or lymph nodes contain recognizable donor cells. There is, therefore, a very marked variability in the degree of injury to the host tissues—a variability that might well be correlated with the degree of tolerance elicited by the cells in the first place.

LUCIE ADLISBERGER (*Montefiore Hospital, New York, N. Y.*): I have two questions for Korngold. First, when human tumors are grown in hamsters, will they differ from one generation to the next? Second, if different parts of a given tumor, which could be either necrotic or "healthy" portions of tumor tissue are used for the extraction of antigen, would similar antigenic preparations result?

LEONHARD KORNGOLD (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): The number of generations that human tumors were grown in rats or hamsters varied, but all of them were grown more than 20 generations, some of them for as many as 100 generations. As yet, no differences have been observed between the early transfers and the later ones.

As for the second question, when a tumor or any other tissue is divided into several parts and the sections extracted at different times, preparations of the same antigenic composition will be obtained, quantitatively and qualitatively.

LESLIE BRENT: I should be interested to learn whether Korngold has had the opportunity to compare tumors freshly removed from patients with those tumors that had been passaged in the rat for many generations.

LEONHARD KORNGOLD: Yes, we had the opportunity to compare the antigenic composition of tumors that were just removed at surgery with the antigenic composition of tumors grown in rats. We demonstrated that four antigens used in this study were absent from most of the human tumors that were grown in these animals. This raised the question as to whether these tumors were deficient in these antigens from the outset or whether they became so after transplantation. One such tumor was deficient after the third transplantation in cortisone-treated animals. As yet, we have been unable to test the original tumors prior to their transplantation.

EUGENE DAY (*Roswell Park Memorial Institute, Buffalo, N. Y.*): When you say that these tumors are deficient in antigens, do you actually mean that the extracts prepared from them are deficient?

LEONHARD KORNGOLD: That is correct.

PAUL H. MAUREK (*University of Pittsburgh, Pittsburgh, Pa.*): I should like to make some comments upon antigenic competition, which has been mentioned. There may be a pitfall involved here; namely, the fact that in your mixture you are injecting varying concentrations of different antigens.

It might well be that the globulin that is injected in the smallest amount is eliminated first, and antibody to it is detected in the serum. However, the albumin that has been injected in the highest concentration may not have been eliminated by that time. It is somewhat dangerous to base the concept of

antigenic competition upon a single bleeding time. W. O. Weigle, in our laboratory, has demonstrated that the guinea pig, which eliminates rabbit serum proteins exceedingly slowly, presents an odd mixture of immune responses. It also has been shown that the time of bleeding is very important in the interpretation of this phenomenon.

LEONHARD KORNGOLD: I understand, of course, that the phenomenon which we term "competition of antigens" is not understood as yet. We use the term solely for lack of a better one.

NORMAN MOLOMUT (*Waldemar Memorial Research Foundation, Brooklyn, N. Y.*): The question that I wish to ask Brent refers to his report that the injection of donor tissue into newborn mice resulted in acquired tolerance to it. All of us are familiar with the fact that the injection of normal or neoplastic tissue will result in acquired tolerance in adult animals. Kaliss and our colleagues have demonstrated this, and we have some unpublished experiments that show that we can obtain tolerance to normal visceral tissues. The question that I ask is based on an interesting observation made in the course of our current experiments. We have found that when we induce tolerance to a tumor graft by the injection of whole-tumor tissue that produces titrable antibody in the recipient mice, and if the surviving graft is transplanted directly into normal adult mice of three strains that are unrelated genetically to either the donor or the recipient strain, such a tumor graft will grow in serial transplantation passages in these animals. This indicates that the tumor itself was affected when transplanted to the strain that was treated with lyophilized tumor to induce tolerance. My question, therefore, is this: Have you, in your work with normal skin, found that the skin graft that survives is altered in the normal host, and is thereby capable of surviving in normal adult mice of strains other than that of the donor?

LESLIE BRENT: First of all, I should like to make a distinction between acquired tolerance, on the one hand, and the phenomenon of enhancement of tumor growth, as described by Snell, Kaliss, Molomut, and their colleagues, on the other. Acquired tolerance of tissue transplants can only be achieved by the injection into embryos or newborns of material containing the active transplantation antigens; the as yet immunologically immature tissues of the host must be confronted with the complete antigenic stimulus. The enhancement of tumor growth may, however, be brought about by the inoculation of adult animals with tissue preparations in which the transplantation antigens have first been inactivated or modified (by lyophilization, for example). In this case a modified antigenic stimulus is presented to animals that are perfectly capable of normal immunological activity. There are several other important differences, although there is not the space here in which to cover them fully. For example, enhancement apparently can be brought about by the prior injection of antisera, a fact that suggests a rather different kind of causal mechanism. Also, we have recently shown that enhancement represents a temporary weakening of the immune response, rather than its complete suppression, which again contrasts with what we know about acquired tolerance. It might well be that these two (and other) phenomena are related, but until there is

clear-cut evidence to that effect it may be more illuminating to think of them as distinct problems.

As far as adaptation of the graft is concerned, we have every reason to believe that, in the case of skin grafts on tolerant mice, adaptation does not occur. This has been shown to be so in a number of ways. For example, if grafts that had been in residence on tolerant hosts were transplanted to normal animals of the host strain, they were destroyed in much the usual way. Conversely, if the graft were retransplanted to mice of the donor strain they were accepted. These simple experiments suggest very strongly that the specificity of the grafts had remained unaltered, and the same principle has been established with a solid mammary carcinoma. These conclusions are supported conclusively by the fact that both immune and nonimmune lymph node cells, when injected into tolerant hosts, will cause the destruction of the previously tolerated graft. Consequently, in our system, skin grafts and solid tumors do not appear to undergo adaptive changes as the result of residence in a foreign host.

On the other hand, Koprowski, elsewhere in these pages, reports very different experiences with ascites tumors: these do appear to undergo antigenic changes that result in a striking loss of strain specificity although, as far as I know, he is still uncertain as to whether these changes are caused by cellular transformations or by selective forces.

RENATO DULBECCO: This was an extensive and critical analysis of the problem of virus assay in the cases in which the response of the host varied considerably from individual to individual. It is, of course, a very difficult problem whereas, in the case in which the individual differences in the host can be neglected, the problem is much simpler. In the latter case the distribution of the affected individuals is the Poisson distribution.

EDWARD A. ECKERT (*State University of New York, Downstate Medical Center, Brooklyn, N. Y.*): Dulbecco has indicated surprise at the divergent results obtained with two related viruses—those producing myeloblastosis and fowl sarcoma. It is relevant that both are tumor viruses, and that the usual host is the chicken. However, the extrapolation of results from one particular virus to another must be done with caution.

As an example, in our experiments we have used two different strains of the leukosis virus, strains generally considered as related to one another or even as variants. Comparisons on the basis of infectivity relationships, immunology, enzymatic activity, and so on, all disclosed definite differences in their properties as noteworthy as the similarities.

Moreover, there is little opportunity to compare the results under identical experimental conditions. The studies presented here by Rubin utilized the membrane of the fertile egg in direct contact with both antibody and virus. No experiments using the chicken are reported. The studies on leukosis were limited to the inoculation of susceptible chickens, the only host system now available. Despite the supposedly more ideal system used in the study of the sarcoma virus, the antibody to normal chick tissue exerted a greater effect on leukosis infectivity when measured in the chicken. Beard, elsewhere in these pages, discusses his attempts to approach the experimental conditions of Rubin in a study of the leukosis virus.

Rubin has mentioned the possibility of the clumping of virus particles as possibly influencing neutralization. The materials used in all neutralization studies of the leukemia virus were observed directly in the electron microscope, and no evidence of clumping was found. However, it was possible to produce clumping and precipitation of the virus of leukemia by immune sera if considerably greater concentrations of the components were used.

ALBERT H. COONS (*Harvard Medical School, Boston, Mass.*): I should like to ask W. H. Taliaferro whether the X irradiation of HeLa cells abolishes their ability to restore antibody formation.

W. H. TALIAFERRO (*University of Chicago, Chicago, Ill.*): We have not performed any experiments with X rays on HeLa cells or any similar materials.

Part IV. Host-Cell Response

CYTOTOXIC EFFECTS OF ANTITUMOR SERUM*

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There are several ways in which cells can be damaged or killed by immunological processes. For example, there may be more or less specific cytotoxic effects on certain cells, or there may be a primary vascular or mesenchymal reaction with secondary injury to surrounding specialized cells. The purpose of this paper is to summarize these cytotoxic immunological phenomena and, in the light of existing evidence, to show how some of them may be used to alter the growth and metabolism of neoplastic cells.

In general, cytotoxic immunological phenomena can be separated into reactions in which circulating serum antibody is easily demonstrable and those in which it is not. In the latter instances there is considerable evidence that immunological damage to the cell results from substances furnished by certain connective tissue cells, particularly the lymphocyte.¹ Both types of immunological reactions have been studied extensively in relation to tumor immunity. Circulating antibodies to tumors have been demonstrated experimentally and may be transferred passively from one species to another (heterologous antibodies) after having formed in response to injections of either living or dead tumor cells (intact or fractionated). Tumor antibodies have also been studied as they have been formed by the tumor-bearing host (homologous antibodies). These serum antibodies have proved effective in preventing "takes" or in producing cytotoxic effects *in vitro*. On the other hand, various characteristics of tumor "immunity," particularly some of those that are demonstrated when a tumor is transplanted to "foreign" strains, appear to result mostly from a tissue response in which mononuclear cells rather than serum antibodies play a major role.

A brief review of the ways in which normal cells can be damaged immunologically may aid in understanding the cytotoxic aspects of tumor immunity. Several of the model immunological reactions that have been studied in the tissues of man and experimental animals are summarized below.

The Arthus, Schwartzman, and Auer Reactions

The Arthus phenomenon² is characteristic of the type of pathological reaction that develops when a high level of serum antibody is permitted to combine with locally injected antigen, usually a soluble antigen, in tissue.³⁻⁵ When it is severe, it leads to localized destruction of tissue at the site of antigen injection, with little evidence of injury to the body as a whole. In this respect it differs from the generalized vasculitis observed in the "serum sickness"

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type of injury that follows injection of large quantities of a foreign serum.⁶⁻⁹ It is reasonable that the pathogenesis of the serum-sickness reaction and the Arthus phenomenon may be similar, since in both there is prominent vascular injury accompanied by thrombosis and hemorrhage.¹⁰ Although the Arthus reaction is classified frequently with the so-called immediate anaphylactic types of hypersensitivity, it is questionable whether this type of tissue injury is correlated well with anaphylaxis or whether it is immediate.^{11,12} It reaches its pathological peak in terms of size of lesion and severity of acute inflammation at about 24 to 48 hours after antigen is injected locally into a hypersensitive animal. The Arthus reaction probably occurs infrequently in man, but there is reason to believe that it can be produced in the human.^{12, 13} It is easily demonstrated in some species of experimental animals, but not in others.¹⁴ It has been produced in several tissues in addition to skin,¹⁵⁻¹⁶ but it probably would be impractical in the treatment of inoperable tumors because, by definition, the antigen must be supplied locally and extravascularly in large quantities. However, a study of this phenomenon serves to emphasize the fact that the cytotoxic effect of the antigen-antibody combination is both destructive and selective.

The Shwartzman-type reactivity, which also seems to have an important vascular component, has been produced in experimental malignant neoplasms. Several authors have described severe hemorrhagic necrosis in tumors following intravenous injection of the active principles of Shwartzman's phenomenon, such as filtrates of meningococci,¹⁷ *Eberthella typhosa*,¹⁸ or *Escherichia coli*.¹⁹ However, these filtrates are quite toxic to both the host and the tumor.

A method of increasing the localization of a cytotoxic antigen-antibody reaction of the Arthus type in non-neoplastic tissue is illustrated by the Auer phenomenon.²⁰ With this phenomenon, circulating antigen and humoral or cellular antibody are apparently brought together in the tissue in injurious concentrations by means of local inflammation induced by nonspecific chemical, surgical, or infectious injury. In the experiments originally reported by Auer, chemical injury by xylol or surgical injury by laparotomy was used to localize intravenously injected antigen in certain tissues of a previously hypersensitive animal. More recently, Hopps²¹ has utilized this reaction to show that hypersensitivity induces delayed healing of wounds. Furthermore, Kirsner and his colleagues have shown that this type of localization can be used to produce acute experimental colitis in the rabbit.²² There have been many attempts to damage tumor cells in a given species with antibodies produced by another species to certain components of the neoplastic cell or to the whole cell.²³⁻²⁶ In general, these attempts have failed to alter the growth of a well-established tumor, in part at least because the intravenously injected antibody has failed to localize in sufficient concentration in the tumor cell. To our knowledge the Auer reaction has not been used to help localize tumor antibodies in tumor, but it is possible that it would be useful for this purpose. In another part of this paper ways by which this might be accomplished are suggested.

Delayed Allergic Hypersensitivity

There is another type of tissue injury resulting from an immune reaction, the application of which to tumor damage is being studied. This is the delayed type of allergic inflammatory response frequently referred to as the "tuberculin" type of hypersensitivity.²⁷ The characteristics of this reaction recently have been reviewed comprehensively by Lawrence.²⁸ When an animal is infected or otherwise immunized with certain agents, usually bacterial or particulate in character, the tissues of the sensitized animal frequently become reactive to these antigens. Severe focal cell destruction may then develop at the site of injection of the organism or some of its products. The principles governing this cytotoxic pathological response include its delayed and sustained character, the absence of easily demonstrable circulating antibodies, and the relative paucity of hemorrhage and edema as an integral part of the pathogenesis of the tissue reaction.^{1, 27, 29}

The necrosis apparently results from the direct reaction of locally injected antigen with mononuclear cells from the immunized animal. Evidence for this concept has been derived from the observation that the reaction can occur in avascular tissue such as the cornea³⁰ or in explanted bone marrow or spleen derived from hypersensitive animals.³¹⁻³² Lympholysis, the specific lysis of lymphocytes of peripheral blood in tuberculin-hypersensitive animals, has been described both *in vitro* and *in vivo*.³³ Particular support for the importance of intracellular substances in this reaction has come from the experiments of Chase and others demonstrating the transfer of delayed skin hypersensitivity by means of a variety of hematopoietic cells.³⁴⁻³⁶ Under special conditions, an alpha globulin of the serum of hypersensitive guinea pigs has been used to transfer the delayed tuberculin skin reaction passively,³⁷ but the serum must be obtained within forty-eight hours after the injection of tuberculin. This may be related to the phenomenon of lympholysis described above, with the damaged immune lymphocytes releasing an alpha globulin fraction that has the same properties as the intact lymphocyte.

Recent interest in the anti-neoplastic application of delayed hypersensitivity stems from the recognition that several instances of experimental necrotizing allergic reactions in normal tissues are probably examples of this type of tissue response. This appears to be especially true of those produced by the use of adjuvants, particularly those devised by Freund³⁵ in which normal tissue components have been combined with the adjuvants. By this method it has been possible to produce variable local cellular damage in the brain and peripheral nerves,^{39, 40, 41} testis,^{42, 43} kidney, lung,^{44, 45} and, more recently, the thyroid.^{46, 47} A considerable portion of the tissue injury resulting from this type of reaction appears to be characterized by atrophy and mononuclear cell infiltration, with demyelination as a prominent feature of the damage to the nervous system. The relative importance of the cellular, humoral, and vascular components in the pathogenesis of these lesions is in doubt, but there is considerable evidence that many of the reactions fit the characteristics of the delayed type of allergic inflammatory response.⁴⁸ Rose and Witebsky,⁴⁶ however, have em-

phasized a correlation of thyroid damage with serologically demonstrable thyroid antibody concentrations.

Recent study of the factors involved in the resistance of a host to foreign tumor and skin transplants suggests that transplant regression may be related to delayed hypersensitivity.⁴⁹⁻⁵¹ If, for example, a tumor derived from one strain is transplanted to a genetically unrelated strain, it usually undergoes regression after one to two weeks. Lymph nodes that have drained the tumor-implantation site are capable of transferring this resistance to an otherwise sensitive animal. In the case of skin transplants, resistance has been transferred by spleen cells. Passive transfer with serum from such tumor- or skin-resistant animals has not been demonstrated. In point of fact, large amounts of such serum have been reported to enhance the growth of tumor which has been transplanted into a resistant foreign species.^{49, 52} Diffusion chambers have also been utilized to demonstrate the importance of cells in transferring this type of immunity.⁵³ When fragments of tumor were placed in chambers that permitted the passage of protein, but not of cells, and when the chambers were placed in the peritoneal cavities of resistant mice, the tumor was not injured.⁵³ The tumor, however, was killed if the pore size of the chamber was large enough to permit the entry of cells. Thus, a form of delayed cellular hypersensitivity appears to be important in the resistance of the host to homologous tissue transplantation.

Serum Antibodies to Cells and Tissues

The Arthus and "tuberculin" reactions can be contrasted with the types of tissue damage that can be produced by the localization of antisera to normal cells or tissue produced in a second species. The use of such sera has increased our understanding of the experimental counterparts of glomerulonephritis,⁵⁴⁻⁵⁵ nephrosis,⁵⁶ thrombocytopenic purpura,⁵⁷ and hemolytic anemia.⁵⁸ A considerable portion of heterologous antibody to kidney can be localized in the glomeruli following intravenous injection.⁵⁹ The acute glomerular damage⁶⁰ can be intensified by superimposed active immune phenomena⁶¹ or by generalized metabolic alterations.⁶²

Cytotoxic Effects of Tumor Antibodies in Vitro

One can logically raise the question whether tumor destruction can be expected when heterologous tumor antibody is administered to a tumor-bearing animal. Examples of selective immunological damage to tumor cells can be found in the early *in vitro* experiments described by Lumsden.^{63, 64} In an extensive series of experiments, Lumsden illustrated the specificity of certain components of heterologous antitumor serum in producing necrosis of tumor in tissue culture. Using human or mouse cancer fragments as antigenic material and antisera produced in sheep or rabbits by multiple injections of large amounts of tumor tissue, he was able to demonstrate the presence of tumor antibodies in the euglobulin fraction of the antisera. These antibodies, in the presence of complement, produced rapid necrosis of either human or mouse carcinoma cells maintained in tissue culture without causing any evident damage to normal human or mouse tissues.

These observations have been extended recently by a number of investigators who have evaluated the effects of antisera to whole neoplastic cells or to cell constituents.²⁴⁻²⁶ In general, most of the emphasis has been placed on demonstration of serologic specificity of the antisera or on prevention of takes after the tumor cells have been treated *in vitro* with the antiserum. A few workers have attempted to evaluate the histological characteristics of the cytotoxic changes produced.

In 1953, Kalfayan and Kidd⁶⁵ reported a study of the structural changes produced by fresh antiserum from resistant rabbits containing complement-fixing antibodies to a saline extract of Brown-Pearce carcinoma. This antiserum was incubated at 37° C. with a suspension of living Brown-Pearce cells. The changes observed sequentially over a two-hour period consisted of swelling and disorganization of the cytoplasm, followed by vesiculation and rupture of the bulk of the cytoplasmic particles, with rapid and almost complete loss of cytoplasmic basophilia. Nuclear changes were less severe, although the chromatin sometimes clumped and assumed a marginal distribution. In some cells, the nucleoli shrank and lost much of their affinity for basic dyes. These changes occurred only when complement was present.

In 1954, Imagawa, Syverton, and Bittner⁶⁶ reported their studies of the effects of guinea pig antiserum to mouse mammary cancer microsomes in tissue culture. This antiserum, containing antibodies to the mammary tumor agent, caused extensive degenerative changes in the tumor cells, as evidenced by shrinkage of the cytoplasm and by pyknosis and irregularity of nuclear shape. The nucleoli became inconspicuous. Exposure of cultures of mammary tumor cells to normal guinea pig serum or to antiserum developed against mammary tissue lacking the tumor virus did not damage the cells. Control cultures of embryonic mouse intestine or HeLa cells were also undamaged by the specific antiserum. Previous studies by these same workers⁶⁷ demonstrated that the action of antitumor serum required complement and that normal rabbit serum was also somewhat cytotoxic.

More recently, Miller and Hsu⁶⁸ studied the cytotoxic effects of antisera to HeLa cell cultures *in vitro*. By means of phase-contrast microscopy they observed decreased motility, retraction of cell processes, cessation of pericytosis, formation of large vacuoles, increased granularity in the cytoplasm, and marked mitochondrial alteration. Nuclear changes were equally striking and consisted of increased density and decreased size (pyknosis), with apparent increased density of the nucleolus in some cells and disappearance in others. In all cases, 3 to 5 per cent of the cells escaped injury. In these experiments, the antiserum to HeLa cells was frequently equally injurious to normal human cells, although some exceptions were noted. Miller and Hsu recorded no attempts to improve the specificity of their antisera by differential adsorption or elution.

Schrek and Preston⁶⁹ have studied the *in vitro* effects of homologous serum obtained from rats with regressed Bagg transplantable rat lymphosarcoma using phase-contrast micrography. Following incubation with this serum, the lymphosarcoma cells showed degenerative changes consisting of rounding up of the cell, followed by swelling and vesiculation of the nucleus and, finally,

rupture and collapse of the nuclear wall. When the cells were suspended in normal rat serum the pattern of degeneration was different, being characterized by actively changing lobulation and nuclear vacuolization. Tumor cells survived incubation in immune serum for only about one hour, while they survived for twelve hours or more in normal serum. The cytotoxic activity of the immune serum required the presence of complement.

Cytotoxic Effects of Tumor Antibodies in Vivo

The first recorded attempts that we have found of the use of ascites tumors to study the cytotoxic and therapeutic effects of antitumor sera *in vivo* are recorded in the Japanese literature. In 1951, Aizawa *et al.*⁷⁰ reported that repeated injections of anti-Yoshida ascites sarcoma serum (50 to 70 cc. per animal injected intraperitoneally over a 4- to 7-day period) produced "complete cures" in a number of rats. Smaller doses produced vacuolar degeneration and some necrosis of the tumor. There is no indication that complement was present in the serum. Subsequent work indicated that this antiserum was quite toxic and that adsorption with normal rat tissues, although leading to decreased toxicity for the rat, led to decreased cytotoxic effects on the ascites tumor cells.⁷¹

Horn⁷² has prepared antisera to whole Ehrlich ascites tumor cells and to components of these cells in rabbits, and has studied the effects of these sera on the tumor cells both *in vitro* and *in vivo*. Although intraperitoneal injection of tumor-bearing mice with 0.1 ml. amounts of these sera did not change their mean survival time, a cytotoxic effect could be demonstrated if the tumor cells were incubated either with sera against the tumor cell mitochondria or nuclei. Phase-contrast microscopic observation of the treated cells for one hour after treatment revealed no significant change that might explain fully their altered growth when placed in a host.

Our interest in the ascites tumor followed difficulty that other workers and ourselves had experienced in obtaining significant localization of labeled anti-tumor gamma globulin in solid tumors following intravenous injection.⁷³⁻⁷⁶ With ascites tumors we believed that one could avoid the problem of the passage of the large gamma globulin molecule across the capillary membrane into the tumor. Thus, one could study cytotoxic effects *in vivo* with minimal loss of tumor antibody to organs and tissues that are normally quite capable of taking up colloidal substances.

In experiments previously published,⁷⁶ as well as in those that follow, we have utilized whole tumor cells as our antigenic material. Although there may be advantages in the use of cell components or chemical fractions to achieve maximum specificity, one must balance against this the possibility of destroying the antigenic pattern that may be unique to the cancer cell by physical or chemical manipulation. With increasing skill in purification of antibody it may be possible to achieve a comparable degree of specificity with either approach.

There are theoretical advantages in the use of a genetically pure strain of experimental animal and a strictly compatible transplantable tumor or, perhaps, even a spontaneous tumor for studies in tumor immunity. On the other

hand, many transplantable tumors, even in somewhat less than pure-bred hosts, seem to be so malignant that they are more lethal than their counterparts in man. The more one observes the process of cancer in man, the more one is forced to conclude that the disease states that we call cancer represent a remarkably variable spectrum. For example, some carcinomas of the stomach approach the malignant potentialities of the ascites tumors, while others are reluctantly invasive and behave frequently like experimental neoplasms transplanted from one genotype to another. In the light of this variable character of human neoplasia, it seems likely that meaningful experiments may be performed with all kinds of experimental tumors, and that results with unusually vigorous transplantable tumors, even when the host is heterozygous, may have the advantage of "stacking the cards" against undue rosy optimism. For these reasons, in our studies we have chosen well-established transplantable neoplasms that have essentially 100 per cent takes and are rapidly fatal.

Importance of the Vascular Barrier

A fairly potent antiserum to the rat Flexner-Jobling carcinoma was produced in the rabbit by means of a multiple portal immunization regimen.⁷⁶ The gamma globulin fraction of this antiserum was labeled with I¹³¹, using the approach pioneered by Pressman.⁷⁷⁻⁷⁸ When the labeled gamma globulin was purified by the Talmage technique of *in vitro* adsorption and exchange elution with additional antiserum,⁷⁹ there was some degree of specific localization on the tumor cell in the test tube. However, when this labeled material was injected intravenously into tumor-bearing rats, only a small fraction of the labeled antibodies appeared in the solid tumor. While the antibody had 1.5 to 3 times the tumor affinity of normal rabbit gamma globulin, most of the injected antitumor globulin localized in the liver and spleen.⁷⁶ Using the Ehrlich ascites tumor, a similarly prepared, labeled, and purified antitumor (AEA)* gamma globulin was injected intraperitoneally into tumor-bearing mice. In this instance, the antibody combined with the tumor cell *in vivo* so that much higher levels of labeled gamma globulin became attached to the tumor cell as compared with the other tissues studied (FIGURE 1).

These results call attention to one simple but important principle that has been frequently overlooked in studies of tumor immunity; namely, that circulating tumor antibody, although fairly specific and potentially effective, may be of little therapeutic value because of its difficulty in passing through the blood-vessel barriers.

Recent studies⁷⁵ indicate that some additional localization of labeled tumor antibodies may be expected where blood vessels are injured in areas of central tumor necrosis rather than at the periphery, as some have suggested.²¹

Importance of Complement in Cytotoxic Activity

A second important principle that seems evident from results obtained in experiments with the ascites tumor is that the presence of complement† may

* AEA is used to designate *anti-Ehrlich ascites* from this point on.

† Complement refers to undiluted guinea pig serum.

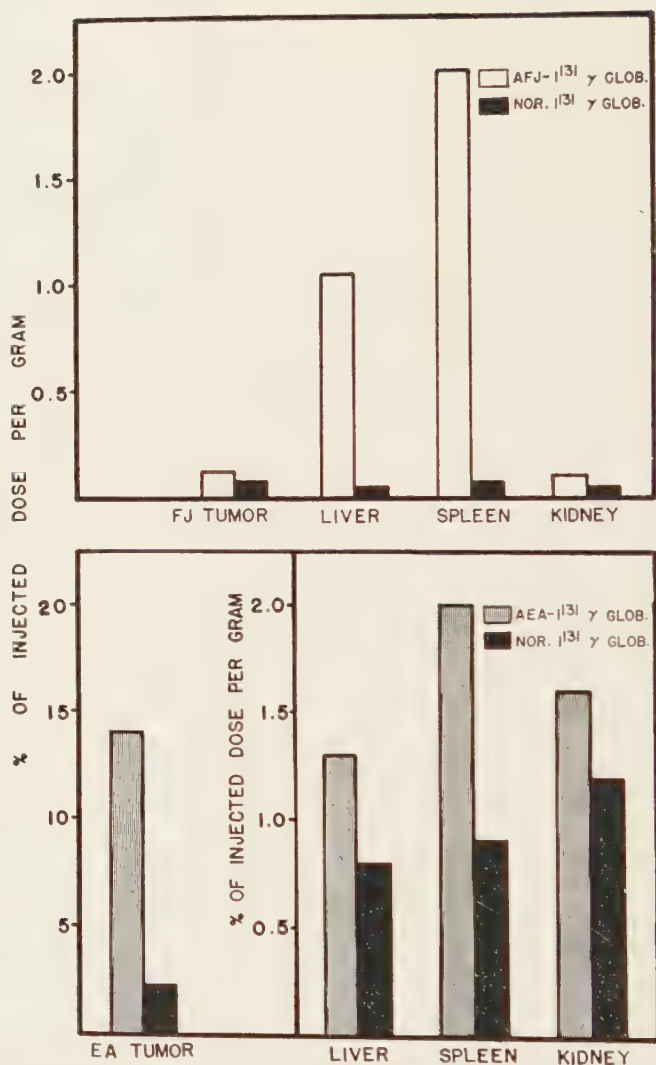


FIGURE 1. A comparison of the *in vivo* tumor localization of similarly prepared and labeled tumor antibodies 24 hr. after injection. The upper graph indicates tumor and normal tissue localization of anti-Flexner Jobling gamma globulin after intravenous injection into rats bearing solid tumors. The lower graph indicates the distribution of 131 -labeled gamma globulin after intraperitoneal injection into mice bearing 4 day ascites tumors. Although the *in vitro* tumor avidity of these 2 preparations was similar, the *in vivo* localization was quite different, depending, apparently, on whether the antibody had to cross blood-vessel walls. The greater localization of normal gamma globulin in normal tissues in the mice may be the result of the larger dose employed relative to body size.

be of critical importance in the production of cytotoxic effects by antitumor serum. The *in vitro* work⁸⁰ with the Ehrlich ascites tumor demonstrated that complement was essential for extensive tumor cell damage, corroborating observations described previously with tissue cultures^{64, 67} and cell suspensions of

solid tumor.^{65, 69} However, Miller and Hsu⁶⁸ found that the potency of the rabbit antiserum to HeLa cells was not heat labile.

We have demonstrated recently the importance of complement in augmenting the cytotoxic and growth-inhibiting properties of AEA antibodies *in vivo*. In these experiments, a pooled lot of antiserum, prepared in rabbits by methods described previously,⁷⁶ has been tested under varying conditions. In general, a single intraperitoneal injection of gamma globulin or antiserum, with or without added complement, was given to groups of CF No. 1 mice bearing 3-day Ehrlich ascites tumors. The dose of antibodies was equivalent to 4.5 mg. of AEA gamma globulin. Complement, when it was employed, was given in a dose of 3 ml. of whole guinea pig serum per mouse. Suitable control injections of saline or guinea pig serum alone, normal rabbit serum or gamma globulin with or without complement, Krebs tumor antiserum, human serum or gamma globulin, and anti-CF No. 1 blood were employed. Groups of animals were sacrificed at various intervals ranging from 12 to 144 hours, at which time tumor weights were determined and samples were prepared for histologic examination.

The results of these experiments, some of which are illustrated in FIGURE 2, demonstrated clearly that AEA gamma globulin alone was almost completely ineffective in retarding tumor growth. AEA globulin plus complement produced marked and reduplicatable damage to tumor cells that was characterized by early agglutination followed later by a decrease in tumor weight. As might be expected, 0.86 per cent NaCl in a comparable volume produced little change in the growth of the ascites tumor (FIGURE 2). Complement alone or normal rabbit gamma globulin plus complement also had little or no effect on tumor growth.

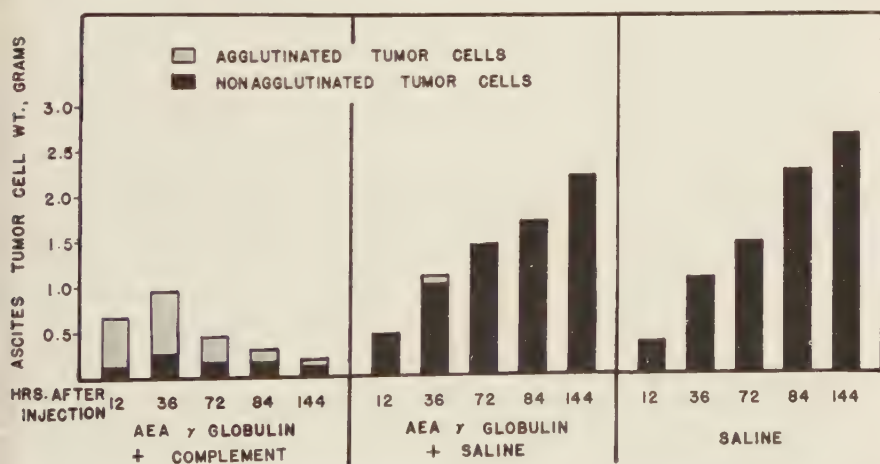


FIGURE 2. Although AEA gamma globulin with or without complement had similar *in vivo* avidity for tumor cells, the graph demonstrates that only the AEA gamma globulin plus complement produced marked agglutination of tumor cells and reduction in tumor weight.

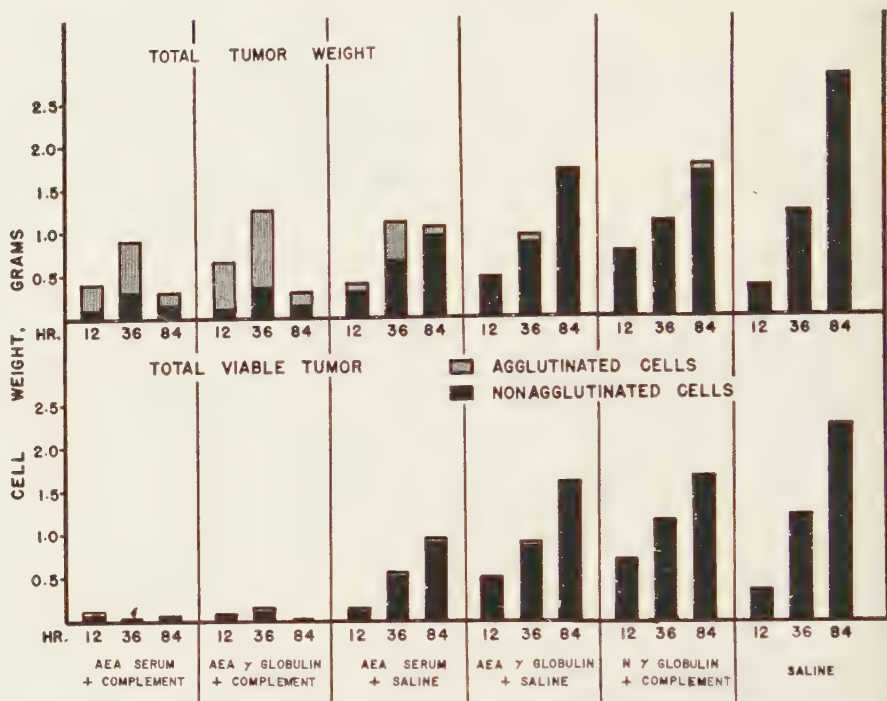


FIGURE 3. The effects on tumor weight produced by several serum and gamma globulin preparations are compared. Greater cytotoxic activity is evident in the lower graph when the tumor weights are corrected by microscopic evaluation of the agglutinated and nonagglutinated tumor, especially in the groups receiving AEA serum, AEA gamma globulin, and AEA serum plus complement.

The apparent importance of complement is further demonstrated in FIGURE 3, in which it is evident that AEA serum, while more effective than AEA gamma globulin in retarding tumor growth as judged by agglutination and tumor weight, is not as effective as AEA serum plus complement. Since the effects on growth and agglutination were similar when either AEA gamma globulin or AEA serum was administered with complement, it seems likely that complement was responsible for most of the increased damage when whole serum was employed instead of gamma globulin. The results from these *in vivo* studies combined with those from previous *in vitro* studies^{64, 65, 67, 69, 80} serve to emphasize the importance of the addition of complement for maximum cytotoxic activity of tumor antibodies. This may be especially true in the interpretation of results of antitumor experiments in the mouse in which there is considerable evidence that normal serum complement activity may be difficult to demonstrate.^{81, 82}

Importance of Histologic Observations

The third principle that becomes evident from studies of the effects of heterogeneous antibodies on the Ehrlich ascites tumor is the importance of micro-

scopic observation in the interpretation of cytotoxic effects. We have referred already to the gross alteration in tumor weight following treatment with various combinations of serum, gamma globulin, and complement. Detailed histological evaluation of this same material led to findings that might otherwise have been unsuspected had only changes in the total tumor weight been considered. For these studies we employed the methyl green pyronine stain that distinguishes ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) under the conditions used (FIGURE 4). Feulgen-fast green was also used for evaluating nuclear changes (FIGURE 5). General histological study revealed the fact that essentially all of the agglutinated tumor was in some stage of cellular degeneration or was necrotic (FIGURE 6). The knowledge that agglutinated tumor is damaged irreversibly helps one discern the greater cytotoxic effect of AEA gamma globulin or serum plus complement as compared with the effect of AEA serum alone (FIGURE 3). Microscopic study of the nonagglutinated tumor also showed that the gross tumor weight was a misleading measure of cytotoxic damage. This was particularly true of tumors taken from groups receiving AEA gamma globulin or serum combined with complement. Much of the nonagglutinated tumor in these groups showed cellular degeneration or necrosis, and there was a variable degree of inflammatory cell infiltration that would lead to false interpretation of tumor weight data (FIGURE 7). As can be seen in FIGURE 3, correction of the weight values for dead cells, erythrocytes, and inflammatory cells demonstrated much greater cytotoxic damage than might otherwise have been revealed.

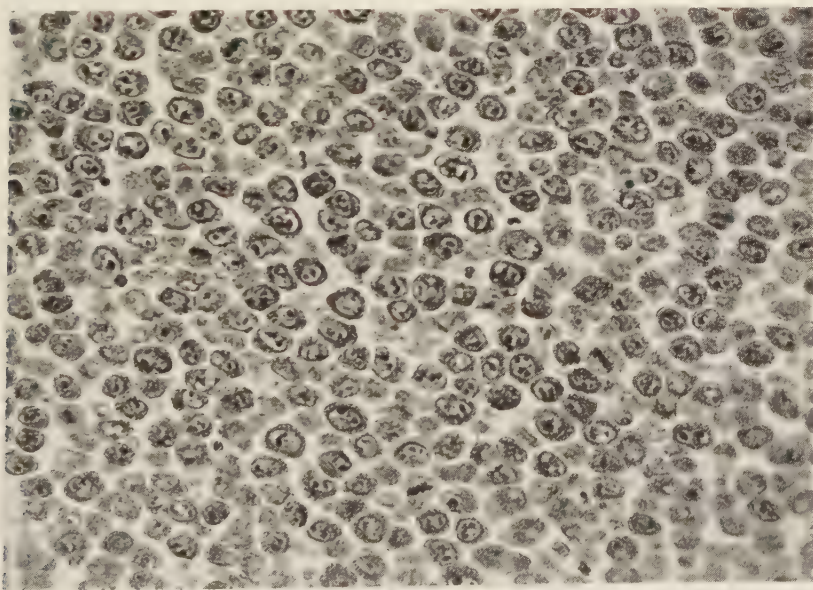


FIGURE 4. Appearance of Ehrlich ascites tumor 7 days after transplantation and 84 hr. after the intraperitoneal injection of 0.86 per cent NaCl . Note the uniform cell size, numerous mitoses, and the pyroninophilic (RNA rich) cytoplasm. Stained with methyl green pyronin. $\times 375$.

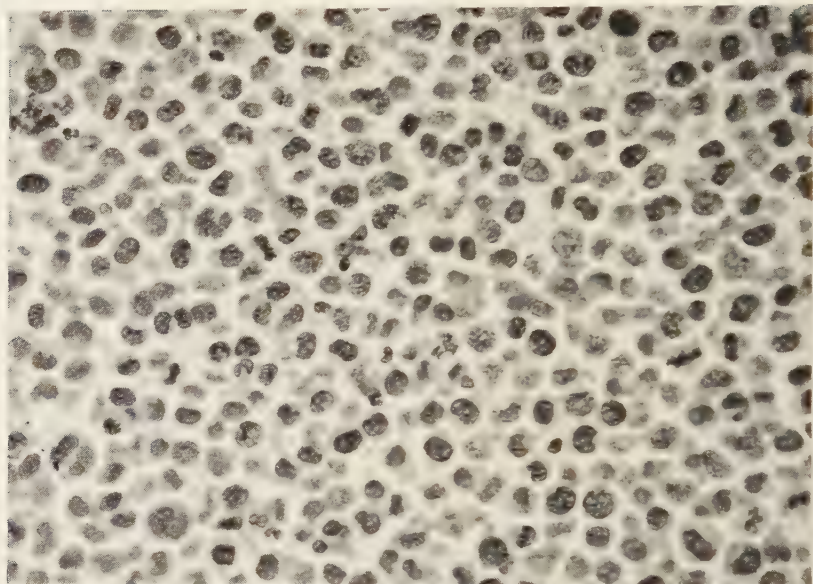


FIGURE 5. Appearance of Ehrlich ascites tumor 7 days after transplantation and 84 hr after the intraperitoneal injection of 0.86 per cent NaCl. The nuclei are rich in chromatin (DNA), and mitoses are common. Feulgen-fast green stained. $\times 390$.

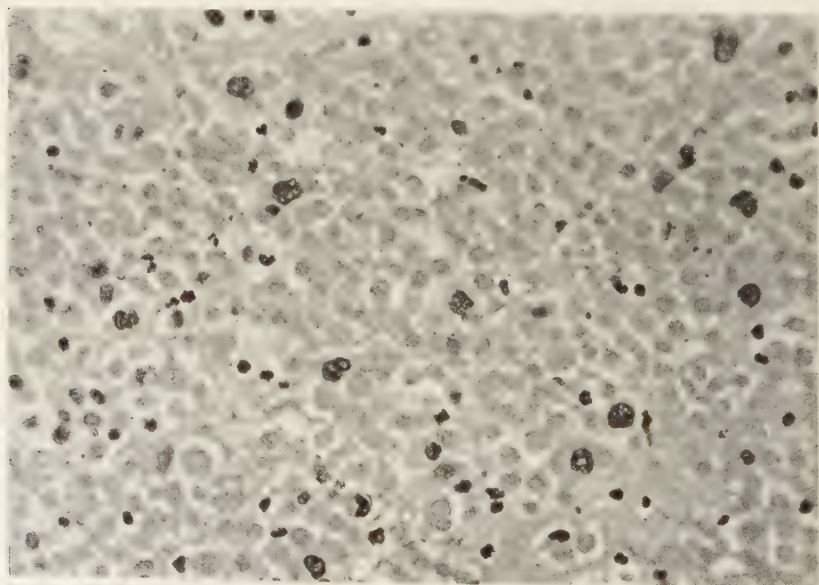


FIGURE 6. Typical appearance of agglutinated Ehrlich ascites tumor 36 hr. after the intraperitoneal injection of 4.5 mg. of MEA gamma globulin combined with 3 ml. of complement. Most of the tumor cells are obviously dead, with nuclear destruction and loss of RNA from the cytoplasm. Feulgen-fast green stained. $\times 375$.

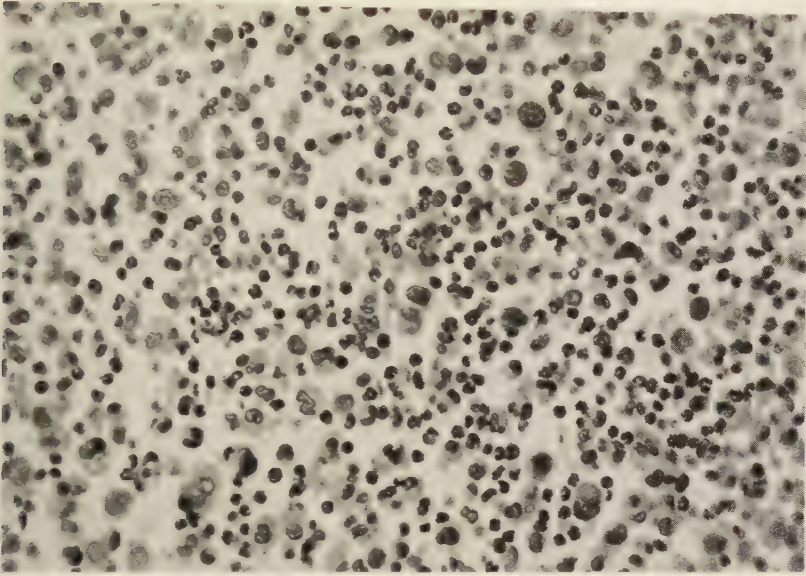


FIGURE 7. Usual appearance of nonagglutinated Ehrlich ascites tumor 36 hr. after the intraperitoneal injection of AEA gamma globulin plus complement. Only an occasional viable tumor cell remains. Most of the cells are polymorphonuclear leukocytes and mononuclear inflammatory cells. Mitoses were virtually absent. Feulgen-fast green stained. $\times 280$.

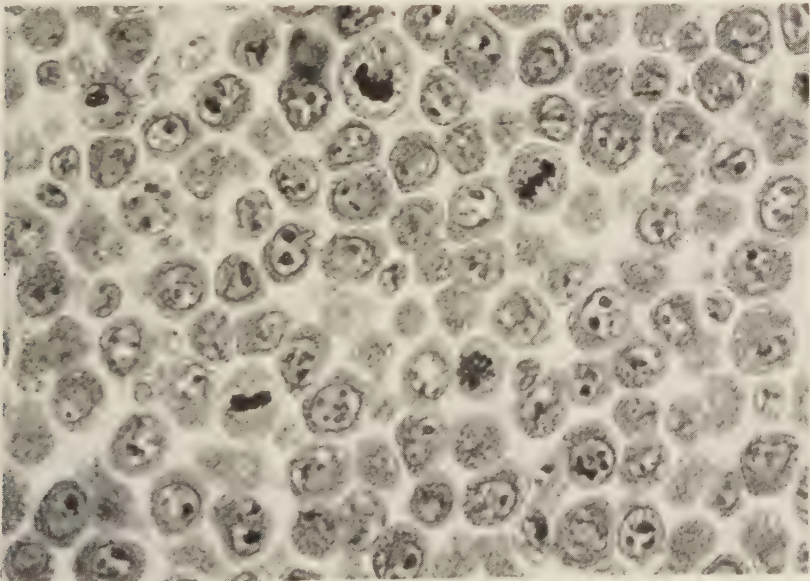


FIGURE 8. Ehrlich ascites tumor 4 days after transplantation and 12 hr. after the intraperitoneal injection of 3 ml. of 0.86 per cent NaCl. Note the deeply staining cytoplasm, the living cells, and the distinct nuclear and nucleolar structure. Methyl green-pyronin stained. $\times 650$.

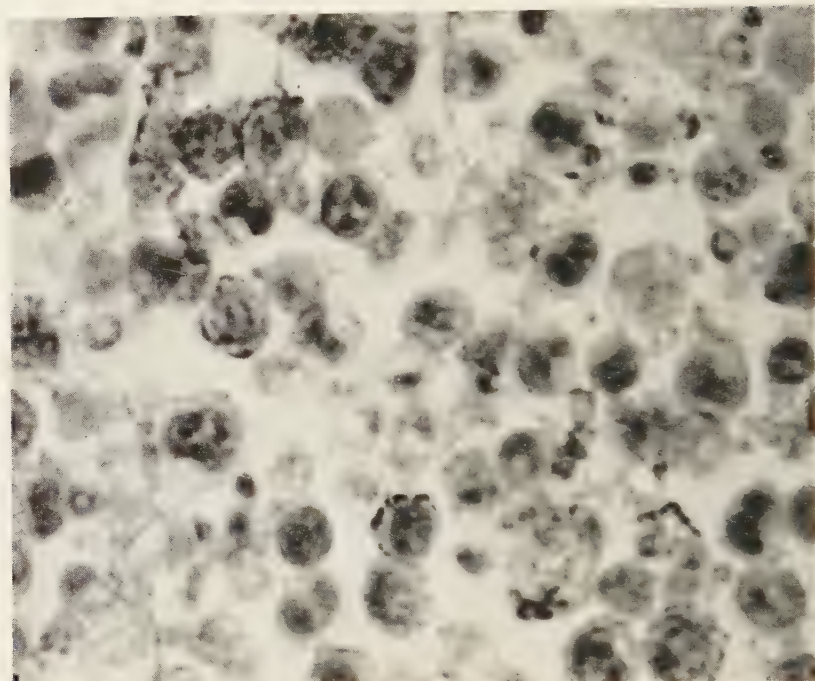


FIGURE 9. Agglutinated Ehrlich ascites tumor cells from an animal sacrificed 4 days after transplantation and 12 hr. after the intraperitoneal injection of 1.5 mg. AEA gamma globulin plus 1 ml. of complement. Note the decrease in cytoplasmic RNA staining, with clumping of pyroninophilic material at the cell membrane. In general, the nuclei are smaller and denser. Methyl green-pyronin stained. $\times 1000$.

Further study of the cytological changes following treatment with AEA gamma globulin and complement was undertaken. Within 12 hours after the intraperitoneal injection of 1.5 mg. of AEA gamma globulin plus 1 cc. of complement into an ascites tumor-bearing mouse, the usual appearance of the Ehrlich ascites cell (FIGURE 8) was markedly altered. There was loss of stainability of cytoplasmic RNA (FIGURE 9). After 36 hours, the cytoplasmic nucleoprotein appeared to be completely absent and the nucleoli no longer stained (FIGURE 10). With 3 times the quantity of AEA gamma globulin and complement, the changes were much more rapid. At 12 hours, many of the cells were completely necrotic, with virtually no mitotic figures. The injured cells that remained (FIGURE 11) had undergone severe nuclear changes consisting of shrinkage and increased density (pyknosis). At 36 hours, the few viable cells remaining consisted principally of unusually large cells with large nuclei containing multiple prominent nucleoli (FIGURE 12). Similar types of cells have been observed following the transplantation of a rat ascites tumor in the mouse.⁸¹ The apparent survival of these polyploid cells is of interest in view of Hauschka's recent observation of the immunoselection of polyploid forms in ascites tumors.⁸¹ Hauschka has called attention to the 3 to 5 per cent of ascites cells that are tetraploid and that seem to survive transplantation into

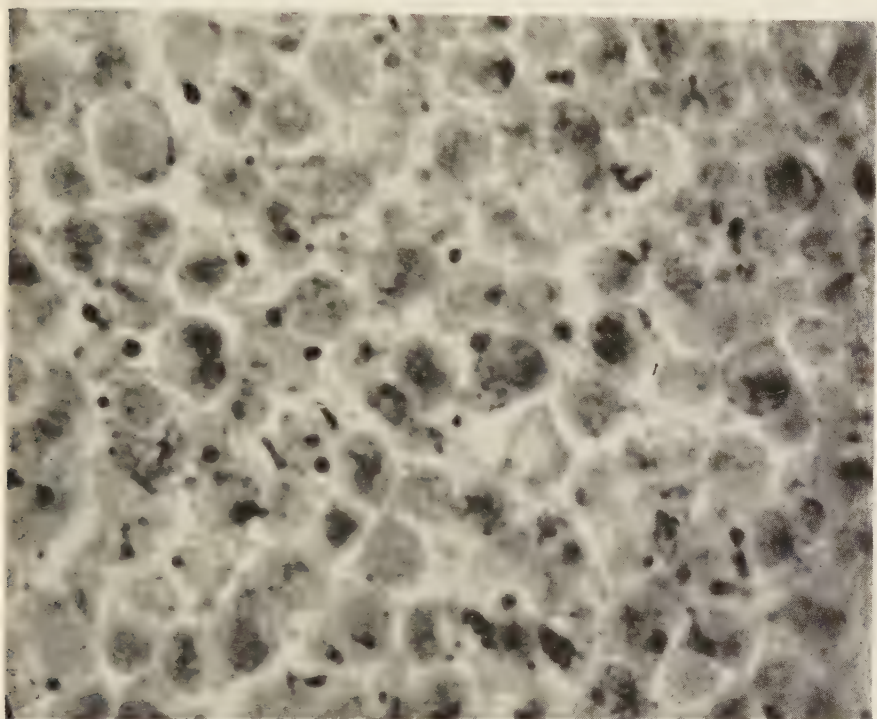


FIGURE 10. Agglutinated Ehrlich ascites tumor cells from an animal sacrificed 5 days after the intraperitoneal injection of 3 mg. of AEA gamma globulin plus 2 ml. of complement. There is almost complete loss of cytoplasmic pyroninophilia (RNA), and methyl green staining material appears in the cytoplasm (DNA). Nucleoli are not apparent, and the nuclei appear to be undergoing karyorrhexis. Methyl green-pyronin stained. $\times 1000$.

a resistant animal. Similarly, in Miller and Hsu's experiments,⁶⁸ 3 to 5 per cent of the tumor cells survived the treatment with ascites tumor antiserum. In the present study we estimated that 1 to 2 per cent of the original tumor was able to withstand the treatment with AEA serum (or gamma globulin) plus complement, and we believe that these were predominantly polyploid forms.

Metabolic Injury

During the study of the localization of the AEA gamma globulin, we undertook to investigate the mechanisms whereby antibody plus complement injured the cell.⁹⁰ Using the neotetrazolium method,⁸⁵ and by direct Warburg measurements, the cells treated with AEA gamma globulin plus complement were unable to utilize either their endogenous substrates or exogenous glucose. They were found to be capable, however, of utilizing succinate. In all experiments, AEA gamma globulin alone produced no evident metabolic alteration in tumor cells. Furthermore, normal rabbit gamma globulin mixed with

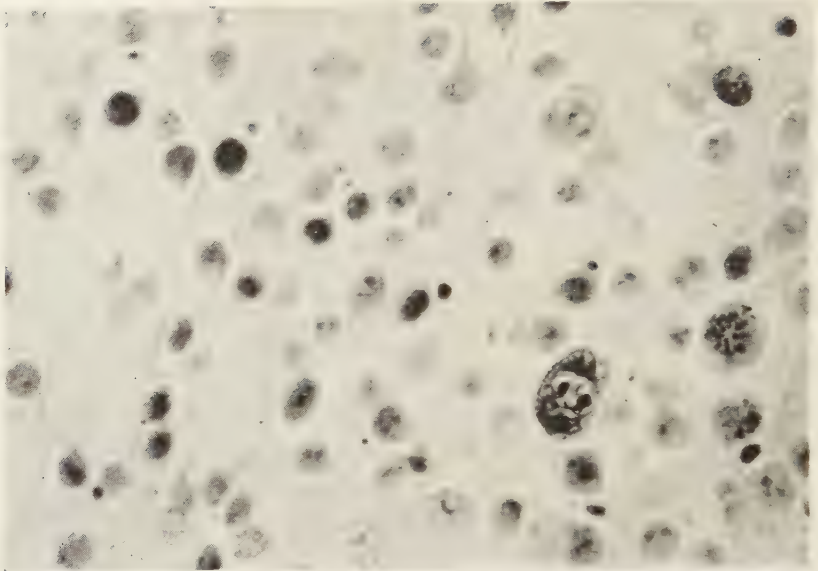


FIGURE 11. Agglutinated Ehrlich ascites tumor cells from an animal sacrificed 4 days after transplantation and 12 hr. after the intraperitoneal injection of 4.5 mg. of AEA gamma globulin combined with 3 ml. of complement. There is much greater evidence of cytotoxic damage as compared to the cells examined after the same interval from animals receiving $\frac{1}{3}$ of this dose (FIGURE 9). Only a few recognizable cells remain. Most of the cells show severe nuclear and cytoplasmic destruction. Methyl green-pyronin stained. $\times 600$.

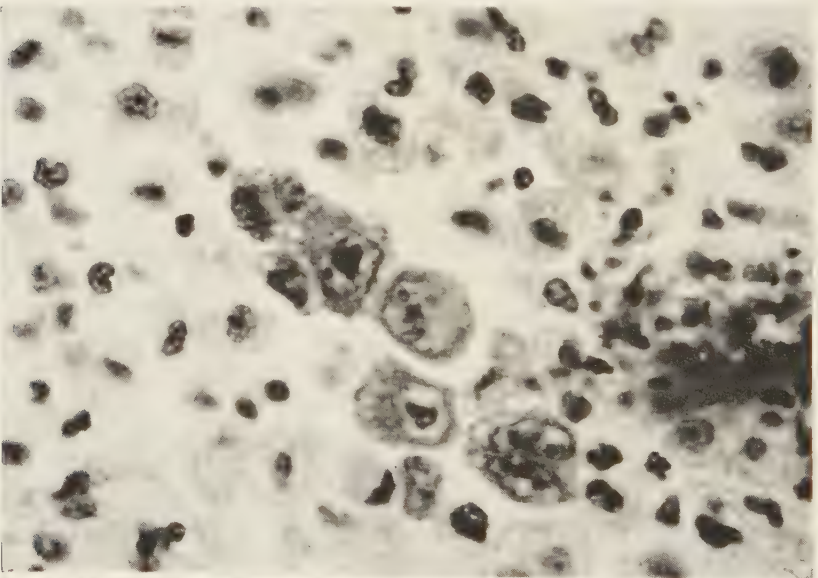


FIGURE 12. Agglutinated tumor from an animal that had received the transplant of an ascites tumor 5 days earlier and that had received 4.5 mg. of AEA gamma globulin and 3 ml. of complement 36 hr. before death. Only a very few tumor cells remain, and these are very large cells with large nuclei and prominent nucleoli. The surrounding cells are mostly macrophages and polymorphonuclear leukocytes. Methyl green-pyronin stained. $\times 600$.

complement did not show an effect on dehydrogenase activity as judged by neotetrazolium reduction, nor were changes in supravital staining observed.

At present it is difficult to be certain whether the failure of these treated cells to metabolize these energy sources indicates a specific cytotoxic damage to the glycolytic system or is only a secondary sign of cell death. Vital staining of the treated cells suggested that these cells were nonviable, at least as evidenced by nucleoprotein dissociation.⁸⁶ Further support for the deduction that these treated cells were dead was their failure to grow on implantation into normal mice. Nevertheless, it is difficult to ascertain when they died relative to the metabolic defects observed *in vitro*, and the mechanism of injury and death may be rather unusual. Kalfayan and Kidd⁶⁵ were unable to reproduce cytologic changes of the type observed following *in vitro* treatment with anti-Brown-Pearce carcinoma serum using a variety of physical and chemical agents. They concluded that in no instance were changes that resembled those seen following treatment with antibody and complement induced.

In all experiments, complement alone produced no evident metabolic alteration in the cell, and heating the mixture of AEA gamma globulin and complement to 56° C. for 30 minutes abolished the metabolic effects.

Specificity and Indications for Further Research

The results at present, then, indicate that under the conditions of our experiments a major portion of the intraperitoneally injected AEA antibody attaches to the tumor cell and that, if complement is present, severe cytotoxic damage results. We have not yet determined adequately to what extent we are dealing with antibody to the tumor cell or its specific constituents. There is some evidence that the cytotoxic action affects tumors in general, rather than a specific neoplasm or its constituents. Anti-Krebs ascites tumor serum, with or without complement, had almost as marked an effect upon the growth and appearance of the Ehrlich ascites tumor as AEA serum similarly tested. This apparent cytotoxic action of one antitumor serum on a second tumor is compatible with the *in vitro* observations that we have reported previously with I¹³¹-labeled gamma globulin.⁷⁶ These studies showed that a cross reaction of one antitumor serum with a second tumor occurred regularly, and that the labeled antitumor serum always combined with the second tumor in greater amounts than with normal tissues.

It might be assumed that we are studying the effects of antibodies to mouse tissues in general rather than to specific tumor antigens. The selective effects on the tumor cells in our *in vitro* experiments might then be interpreted as the result of the close proximity between antibody and tumor cells. One would still need to explain the greater *in vitro* localization of the labeled, adsorbed, and eluted gamma globulin on the tumor cell as compared with control tissues.⁷⁶ By comparing the effects of the AEA serum with antiserum to normal organs or tissues, we are attempting to obtain further control observations that will help answer this question. Thus far, we have found that antiserum or gamma globulin to CF No. 1 mouse blood is very toxic to the mouse. In surviving mice, however, no striking effects have been observed in the appearance of the tumor cells.

With the indication of some degree of specificity *in vitro* and *in vivo* in our experiments and in those of others, it appears profitable to pursue the study of heterologous antitumor sera further. One possible approach would be to attempt to increase the specificity and cytotoxicity of intravenously injected tumor antiserum by further purification procedures. Recent results reported by Pressman and his co-workers³⁷ indicate that this is a very promising line of investigation, although cytotoxic effects have not yet been reported.

Another possible step would be to alter the vascular bed of the solid tumor so that the passage of the large globulin molecule across the capillary membrane would be facilitated. Several possibilities merit investigation in an attempt to increase vascular permeability in a tumor. These might be considered as modified or inverted Auer reactions, and one, the last to be discussed, is an example of one application of the principles of delayed allergic inflammation.

One might attempt to induce increased vascular permeability in the region of a solid tumor by irradiation prior to administration of tumor antibodies so that greater differential localization of the globulins could be attained. As a second possibility, it might be practical to alter the tumor vascular bed by means of inflammation produced by tumor specific viruses. Either of these procedures would be similar in mechanism to the Auer phenomenon. A third method that might stimulate increased localization of intravenously injected antibody in a solid tumor is the production of a delayed allergic inflammatory reaction within the tumor prior to injection of heterologous tumor antiserum.

At present, we are actively investigating this third proposal. At the same time, we are studying the possibility of producing injury to a growing solid tumor by using only the delayed hypersensitivity cellular response by the host to its own tumor. The fact that this mechanism produces striking destruction of some types of normal cells³⁹⁻⁴⁷ suggests that it might be applicable to some types of neoplasms. In point of fact, it may operate spontaneously to limit the spread of certain malignant tumors in man.⁴⁸ It is possible that this type of local tissue damage will induce a mild inflammatory reaction that will facilitate localization of intravenously injected tumor antibodies in solid neoplastic tissue.

The Combined Approach

Attempts are being made to induce autoimmunity to the Brown-Pearce rabbit tumor by removing one of a pair of well-established tumors that were originally implanted nearly simultaneously. The resected tumor is homogenized with Freund's adjuvant, heated to 58° C. for 15 min., and injected as multiple intramuscular depots into the same animal. A similar type of experiment has been performed in rats bearing the Jensen sarcoma. Because of some promising preliminary results, this approach is being extended to the critical phase of intravenous injection of anti-Jensen serum plus complement after an autoimmune state has had time to develop.

This approach receives theoretical support from recent work by others. Gorer⁵² has reasoned that humoral antibodies help promote the delayed hyper-

sensitive response in genetically determined resistance to tumor transplants. He suggests that such resistance is related to the presence of "immune" lymphocytes that inhibit tumor growth by local infiltration of the tumor tissue. Applying the concept of lympholysis as established in tuberculin hypersensitivity,³³ one might expect that these antitumor lymphocytes would be destroyed in the presence of tumor antigen. The lymphocyte seems to be of major importance in supplying cytotoxic substances near the tumor cell, and *serum* antibody may protect the lymphocytes by reducing the antigen concentration intravascularly and at some distance from the tumor.

It seems likely that a combined approach to immunological cytotoxic damage, utilizing both circulating and cellular antibodies to tumor, may be promising and ultimately may reconcile what have seemed to be two divergent concepts concerning tumor immunity.

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CYTOTOXINS AND CYTOTOXIC ANTIBODIES

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Consideration of the proposition of anticancer antibodies suggests that they might be important for two reasons: (1) insofar as they might be useful in cancer therapy, and (2) for what they might reveal about cancer cells or processes. The newcomer to the field of cancer immunology is struck by the curious situation that, while interest in cytotoxic antibodies has been almost continuous from the origin of cancer research, years of investigation have neither relegated this phase of cancer immunology to obscurity nor established its investigative value. Bashford, Murray, and Haaland¹ in 1908 concluded that "As regards the hope of a practical outcome . . . we consider that it is not at present to be sought in the direction of a curative serum. . . ." Forty-four years later, on the basis of his review, Hauschka² could offer little more hope. The conference on which this monograph is based offered an appropriate occasion to see whether research done since 1952 gives more or less reason for enthusiasm, from either the clinical or experimental point of view, for the use of antiserum to mammalian cells.

The cytotoxins to be considered are limited to those found in serum or body fluids. The title of this paper was assigned deliberately to emphasize a distinction that has not always been made in the literature. It is now a common experience in cell culture that supposedly normal serum can be variably toxic to animal cells. The fact that such toxicity can depend more on the batch of serum than on the donor species complicates the question of adequacy of controls for experiments involving cytotoxic antibody. The latter substance is defined here as a specifically modified serum protein, produced in response to exposure to an inducing substance, that is capable of combining specifically with the inducing substance (antigen). With the complex antigen-antibody systems present in cell-serum mixtures, experimental definition of antibody is difficult unless resort is made to a preparation of purified inducing antigens and reacting antibodies. For example, in an experiment recently performed, aliquots of a suspension of human epithelial cells were mixed respectively with preimmunization and postimmunization monkey sera. When samples of the reaction mixtures were assayed for viability of the cells, the immune serum was shown to have reduced the viability of exposed cells. The tests were then repeated with inactivated test sera mixed with human complement: the toxic effect of the immune serum on the cells was nullified. Presumably something other than antibody was involved. Other experiments on the suitability of various animal sera for propagation of different types of human cells have shown that exposure of cells to serum in high concentration can result in cellular multiplication, maintenance without multiplication, or slow or rapid death. The same sample of serum may produce some or all of these effects with different cell types. Valid demonstration of anticellular antibodies requires at least: (1) testing of adequate numbers of preimmunization and postimmuniza-

tion sera, not just representative pools; (2) testing of control systems with inactivated antiserum and inactivated complementary serum; (3) independent testing of control systems with antiserum and complement alone; and (4) verification of specificity, if only by showing that particular cells absorb the toxic material from serum. It would also be preferable to show that the concentration of the toxic factor in the serum of immunized animals increases with repeated exposure of the animals to the inducing antigenic material.

Reports of serum cytotoxins published in the more commonly available journals since the last comprehensive review of cancer immunology have been surveyed with the above considerations in mind, in an attempt to answer some specific questions about cytotoxic antibodies.

Can Cytotoxic Antibodies Be Produced?

In some instances, serum cytotoxins have been demonstrated under circumstances that seem to exclude antibody action. The history of the effect of normal guinea pig serum on certain mouse lymphomas, particularly the Gardner lymphosarcoma of C3H mice, worked out by Kidd, Todd and Kauffman,³⁻⁷ provides a fascinating example. It was found that normal guinea pig serum would protect C3H mice passively against the 6C3HED lymphosarcoma and A mice against Lymphoma II, or would temporarily inhibit Murphy-Sturm lymphosarcoma in rats; however, no effect was exerted against mouse carcinomas or sarcomas. Both horse and rabbit sera were inactive. The activity of the cytotoxin against the C3H lymphoma was enhanced by reinforcement with specific rabbit antiserum. No inhibition of lymphoma cells resulted from *in vitro* exposure, although *in vivo* cytotoxic effects could be observed. Later experiments revealed the fact that the normal serum did not protect against AKR lymphomas in AKR mice, but did enhance the cytotoxic activity of rabbit antiserum to AKR cells. The possibility that the guinea pig serum might be providing complement to activate isoantibodies induced in mice by the 6C3HED cells was discounted by the incapacity of mouse sera that should have contained isoantibodies, if such existed, to enhance the guinea pig serum factor. Further, the serum was no more effective in mice relatively resistant to the lymphosarcoma than in mice relatively susceptible. The guinea pig serum factor also was able to inactivate C3H lymphoma cells selectively when these and AKR cells were implanted on opposite sides of hybrid mice. A similar action of commercial guinea pig serum on rat fibrosarcoma has been observed by Jameson, Ainis, and Ryan.⁸ Other less extensively investigated cytotoxins have been added to the list: for mouse leukemic cells in normal rabbit serum by Werder *et al.*;⁹ for mouse mammary tumor cells in normal rabbit serum by Imagawa, Syverton, and Bittner;¹⁰ and for HeLa cells in normal rabbit serum by Miller and Hsu.¹¹ These few instances of normal cytotoxins found in sera most commonly employed as sources of antibody and complement show the necessity of prior testing before selection of serum-donor species in cytotoxic antibody studies. Further reason for caution is given by the existence of blood-group isoantigens in somatic cells.¹²⁻¹⁴

Cytotoxins that might be or were demonstrably antibody in nature have been elicited in horse, chicken, rabbit, guinea pig, rat, or mouse serum in 15 of

20 reported studies. Most often the affected cells have been lymphoid, but myeloid, epithelial, and fibroblastic cells also have been successfully damaged by antiserum. Susceptible cells have been derived from normal or neoplastic human, rabbit, guinea pig, rat, and mouse tissues. Failures to demonstrate immunopathogenesis involved similar types and origins of cells. In no instance was failure to produce anticellular antibody accompanied by assurance that adequate attention had been paid to quantity of antigenic material used for immunization, course of immunization, and necessity for complement reinforcement of antibody. Considering the wide variety of materials and procedures employed in successful attempts, it is probably true that production of cytotoxic antibody is intrinsically no more difficult than production of antibody to protein antigens in general.

In 5 of the 20 studies considered, cytotoxic action of antiserum was dependent on complement. In 2 studies unequivocal cytotoxic activity was seen when complement was necessarily inactive. These two findings can be reconciled if, as might be expected, cells can be damaged by sufficient antibody without intervention of complement, but the extent and severity of damage are increased by the action of complement. Rigorous evaluation of the role of complement in immune cytopathogenesis is prevented by lack of information on the quantitative relations involved in interaction of cells, antibody, and complement. It should be noted that proper interpretation of immune cytotoxic action must depend on full appreciation of the qualitative and quantitative relations existing among the 3 reagents. It is known for complement fixation generally that the extent and outcome of reaction are determined by relative concentrations of antigen, antibody, and complement, and by the serum sources of antibody and complement. Whether the cytotoxic capacity of antiserum is tested *in vitro* or *in vivo*, possible deficiencies of complement components or incompatibility of complement and antibody could mask or modify the activity of antibody of even high specificity, but low potency. Usually more specific antisera result from courses of immunization that cannot be expected to yield antisera of great potency.

Commonly, the cytotoxic antibody reported appears to have been of the circulating relatively heat-stable type. In one instance only,¹⁵ *in vitro* inhibition of cells required the presence of lymphoid cells from immune animals.

Can Cytotoxic Antibodies Act in Vivo?

In demonstrating *in vivo* cytotoxic action of antibody by passive protection test, Nungester and Fisher,¹⁶ Colter *et al.*,¹⁷ and Gorer and Amos¹⁸ showed that the protective power of antiserum diminished rapidly as the interval between challenge and antiserum administration increased. When Kalfayan and Kidd¹⁹ exposed excised fragments of Brown-Pearce carcinoma to antibody and complement, only peripheral cells were affected. Wissler *et al.*²⁰ were unable to obtain strong localization of passively administered antibody, labeled and specifically eluted, in formed rat tumors, although such antibody readily concentrated *in vivo* on Ehrlich ascites tumor cells. Apparently, in the early stages of tumor formation, antibody cannot diffuse to inner cells, while in later

vascularized stages barriers are interposed by intact capillary membranes. Quantitative relations could constitute a third factor in *in vivo* effectiveness of antibody against formed tumors, because common antigens diffusely distributed throughout the body, even in low concentration per unit mass of tissue, conceivably could reduce circulating antibody levels below the minimum required for progressive destruction of a tumor mass. Although cytotoxic antibody can be fully active *in vivo*, it seems that therapeutic use for one reason or another may be limited to the time when tumor cells in the body have not formed solid foci of growth. This restriction would not preclude use against leukemia, or for postsurgical treatment to reduce the frequency of "second looks."

In What Sense Are Cytotoxic Antibodies Specific?

Antibodies are specific by definition in that they are modified according to the surface configurations of the particular molecular units indicated by the term "antigens." Experimentally, however, with certain exceptions, anti-cellular antisera will contain mixtures of antibodies specific for various parts of the cellular antigen mosaics. Under these conditions antiserum specificity must be derived from the quantitative relations between reacting cellular antigens and serum antibodies. Cytotoxic antisera studied have been surprisingly specific considering that they were produced against whole cells or cellular homogenates, as well as subcellular fractions. At least, as Miller and Hsu¹¹ found, species specificity has existed; at most, as Snell and Favata,²¹ Schrek and Preston,²² and Björklund, Graham, and Graham²³ learned, specificity has extended to cell type within the same species or even tissue. The more expected situation, quantitative specificity, is represented by the findings of Werder *et al.*⁹ with rabbit antisera to leukemic and normal mouse cells; the greatest protection against leukemic cells was conferred on challenged mice by antileukemic serum. Similarly Flax²⁴ observed that, while antiserum to Ehrlich ascites cells was equally effective against cells of both Ehrlich and Krebs ascites tumors of mice, antitumor antibodies were differentially absorbed by tumor cells and normal cells of various types. In a number of cases, specificity of cytotoxic action has been associated with a particular component of particular cells. Kalfayan and Kidd,¹⁹ for instance, showed that immune rabbit sera were cytotoxic for Brown-Pearce carcinoma cells only when the sera contained antibody to the distinctive sedimentable constituent; these antisera were harmless to many normal rabbit cells and to Vx2 rabbit carcinoma cells. Imagawa, Syverton, and Bittner²⁵ observed a cytotoxic action on mouse mammary tumor cell cultures produced by rabbit antisera to mouse tissues containing Bittner virus, but no such action of antisera to tissues lacking the virus. E.L. 4 mouse leukemia was shown by Gorer and Amos¹⁸ to contain an antigen apparently not characteristic of the mouse strain of origin or antigenic in such mice. Whatever the origin of this and other distinctive specificities, they provide evidence that highly specific cytotoxic antisera can exist. To consider specificity in another way, where tests have been made, the combining capacity of cytotoxic antibody has been verified by

depletion of serum activity by exposure to cells. Mountain's²⁶ use of absorption to verify the antibody content of rabbit anti-HeLa serum is exemplary.

Apart from possible clinical applications, the various specificities of cytotoxic antiserum observed by different workers suggest considerable research value of the cellular neutralization test in defining biological differences between cells.

What Effects Do Cytotoxic Antibodies Have on Cells?

Most workers report similar visual evidence of immune cytopathogenesis: rounding and swelling of cells with retraction of normal processes, increasing cytoplasmic granulation and mitochondrial vesiculation, and increasingly severe nuclear damage. Loss of cell viability apparently is gradual. Snell and Favata,²¹ for example, were able to interrupt the process by washing antibody-treated cells, and to repeat the damage by new exposure of the recovered cells to antibody. Actual lysis of cells seems uncommon, although no judgment can be made because complement has not yet received adequate consideration in studies of cytotoxic antibody. The work of Flax²⁴ is notable in providing the beginnings of a biochemical interpretation of morphologic damage to cells. The cytotoxic action of complement-dependent antibody to Ehrlich ascites tumor cells, in the form of purified gamma globulin, was related to loss of cellular capacity to metabolize glucose with retention of capacity to utilize succinate. These findings are valuable also in (1) suggesting a possible application of cytotoxic antiserum to metabolic study of cells and (2) indicating procedures for quantitative assay of cytotoxic antibody.

General consideration of contributions to the study of cytotoxic antibody during the last four years suggests that continued research would be profitable. The possibility of cancer immunotherapy, at least as an adjuvant to surgery, cannot yet be discounted, particularly when possibilities of imparting added specificity and potency to antisera are still being explored.^{27, 28} The use of cytotoxic antibody as an investigative tool for biological characterization of mammalian cells is promising but has barely been attempted. It is apparent, however, that better definition of the quantitative aspects of cellular neutralization is required.

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CYTOTOXIC EFFECTS OF ANTISERA AGAINST HUMAN EPITHELIAL CELLS GROWN IN TISSUE CULTURE

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Although there has been some confusion between inherent cytotoxins in certain animal sera and true cytotoxic antibodies produced by purposeful immunization of experimental animals, the existence of the latter has been well established.^{1, 2} However, there are certain limitations to the use of this immunological procedure, as has been noted by other contributors to this monograph. It is our purpose to present some of the results we have obtained with this technique using antisera prepared against certain tissue culture-grown human epithelial cells as illustrations of these limitations.

Antisera were prepared in rabbits by hyperimmunization with cells grown in media containing either horse serum or homologous rabbit serum. Immunization involved several doses of cell antigen in Freund's adjuvant, followed by several intravenous doses. All tests for cytotoxic effect were carried out in roller-tube cultures of the cell types being tested; 0.1 ml. amounts of antiserum dilutions were added to the 1.0 ml. of maintenance medium on the cultures. The routine maintenance medium consisted of 2 per cent horse serum in Eagle's medium³ unless otherwise indicated. Most of our work has been carried out with an antiserum produced against Wilton Earle's human skin cell line,⁴ but some experiments have utilized antisera to HeLa cells, KB cells,⁵ and human amnion cells.

With all these antisera we have found high titers of both hemolysins and hemagglutinins against A+, B+, and O- human red blood cells. The hemolysin titers have gone as high as 1:800 and agglutinin titers to 1:2400. Since these hemolysins represent potent heterologous cytotoxic antibodies, we have carried out adsorption of our antisera with human A and B red blood cells. In some instances it has required as many as 11 adsorptions using 50 per cent volumes of packed cells before all hemagglutinins have been removed from the antiskin sera.

In general, our results have paralleled those reported by others. When antisera have been inactivated and used without complement, the microscopic appearance of treated cell sheets in roller tubes has been that of rounding of cells, a tendency to granularity of the cytoplasm, and a shrinking of the cell sheet in a way to resemble agglutination. On the other hand, when antiserum was used in the presence of complement, lysis of cells with loosening from the glass surface was more apparent. Titers of cytotoxic effect of antisera were relatively low, usually at 1:40 to 1:80 final dilution against homologous cells. In general, titers against heterologous types of human cells were lower except in the case of human fibroblast cultures that appeared to be readily susceptible to morphologic changes due to antiserum against skin cells, for example. We feel this is probably due to the fact that any change in cell outline of the normally elongated fibroblast is easily detectable as compared to the more rounded epithelial cell. Apparently the compactness of the cells in the cell sheet may

be a factor in the ability of an antiserum to show cytotoxic effects. For instance, human amnion cells that seem to form a closely compact sheet on glass show definite morphologic changes only on the periphery of the sheet even when treated with homologous antiserum.

In preliminary experiments we have found that antibodies to the serum components of either the cells or the tissue culture media do not have any apparent cytotoxic effect. Antiserum against human serum, for instance, has no effect on the cells, even though we know from complement-fixation tests that these human cells do contain certain antigens present in human serum. Likewise there was no difference in the cytotoxic titer of an antiserum prepared against cells grown in horse serum medium and known to contain precipitins and complement-fixing antibodies against horse serum when it was tested against cells maintained in a horse serum or calf serum medium.

In an attempt to make the test of cytotoxic action more sensitive, the titer of antisera added at the time of original planting of cells was compared to that obtained when the antiserum was added to intact sheets of cells. The titers were the same. Of course, this brings up the important problem of criteria of cytotoxic effect. Obviously, morphologic appearance of treated cells or even their detachment from glass does not signify cell death. On the other hand, the ability of treated cultures to grow on subsequent incubation in the presence of growth media merely signifies that there was a minimum number of viable cells left to re-establish the culture. Practical quantitative methods for establishing the exact numbers of viable cells are not readily available. Using antiserum prepared against the ascites form of S37 mouse sarcoma cells, we have attempted to use vital stains as a means to this end. Neutral red on these cells gives poor differentiation, the dye being taken up in such varying amounts that it is difficult to establish an end point. Recently we have used trypan blue to stain the nonviable cells, and here there seems to be sharper differentiation. Percentage of stained cells following treatment of a cell suspension with antiserum correlates well with antiserum dilution. Further experiments by animal inoculation are necessary to determine if trypan blue is really differentiating nonviable from viable cells after antiserum treatment.

TABLE 1 shows the results of a typical cytotoxic antibody experiment in

TABLE 1
CYTOPATHOGENIC EFFECT OF RABBIT ANTISERUM TO TISSUE
CULTURE SKIN CELLS (EARLE)

Cell culture	Inactivated—no complement		Inactivated—complement	
	Unadsorbed	Adsorbed	Unadsorbed	Adsorbed
Skin.....	—	—	160*	<40
HeLa.....	40	10	80	<40
Amnion.....	20	<10	—	—
Fibroblasts.....	80	10	>640	160
Monkey kidney.....	<10	—	—	—

* Reciprocal of final serum dilution.

which antiserum against the skin epithelial cells was used both before and after adsorption with human red blood cells. The cytotoxic action of the antiserum was investigated in the presence and absence of complement in the form of freshly obtained normal guinea pig serum (0.05 ml. per roller tube culture). End points of titrations given in the table represent 2+ end points where destruction of cells evident on microscopic examination was graded from 0 to 4+. Titers of both adsorbed and unadsorbed sera were higher when used in the presence of complement. All titers except that against human fibroblasts were of low order, and there were cross reactions with all human cells tested, but not with monkey kidney epithelial cells. Adsorption of the antiserum with human red blood cells reduced the cytotoxic titer for both heterologous and homologous cells.

Summary

Antisera produced against established tissue culture lines of human epithelial cells have a cytotoxic effect on the homologous cell type, but cross react with other human cell lines in tissue culture, especially with human fibroblasts. Higher titers of cytotoxic action of antisera are obtained if complement is present, but in most instances the titers are of a relatively low order of magnitude. Antisera have high levels of hemolysins and hemagglutinins for human red blood cells which can be adsorbed. However, this adsorption reduces the cytotoxic titer not only for heterologous, but also for the homologous cell type.

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THE NATURE OF THE ANTIGENIC STIMULUS IN TRANSPLANTATION IMMUNITY

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It has recently been demonstrated¹ that tissue cells need be neither viable nor structurally intact in order to elicit transplantation immunity in homologous hosts. Mouse spleen cells suspended in a variety of media can be totally disintegrated by exposure to ultrasonic irradiation without abolishing their capacity to immunize adult mice against skin grafts from the donor strain. It has been shown further that this power to immunize lies solely in the nuclear material of spleen and kidney cells; it is not found in their cytoplasmic components.

The finding that the tissue substances responsible for homologous transplantation immunity are nuclear rather than cytoplasmic is entirely in accord with the well-known lack of tissue specificity both in transplantation immunity and immunological tolerance. For example, adult animals can be immunized against skin homografts by the prior injection of blood leukocytes² or spleen and kidney cells,³ or by the prior transplantation of whole kidneys⁴ provided these are derived from the skin donor or animals of the same inbred donor strain. Conversely, the injection of such cells into *embryos* brings about a state of immunological tolerance of the cell donors' skin grafts in the recipients.⁵ Furthermore, the neonatal injection of mice with spleen cells induces tolerance not only of skin grafts,⁶ but also of ovarian⁷ and adrenal⁸ tissues; and the exchange of blood cells in fetal dizygotic cattle twins leads to tolerance of skin⁹ as well as of kidney tissue.¹⁰ These facts strongly suggested the presence of the same set of transplantation antigens in all the tissues of the body of a single individual, and pointed to the nucleus as the source of the antigenic stimulus long before this could be demonstrated by direct experimental methods.

The above considerations do not, however, apply to mammalian red cells: when carefully separated from the blood leukocytes and injected into homologous hosts, they are unable to elicit an immunity which is directed against grafts of skin^{5, 11} (the fact that other workers have reported very different results with neoplastic tissues^{12, 13} has yet to be explained, and has been discussed elsewhere¹⁴). That mammalian red cells should not possess transplantation antigens is perfectly intelligible in view of the absence of nuclear material in these cells. *Avian* red cells (which are known to be incapable of inducing immunological tolerance of skin homografts⁵) raise an interesting problem in this connection and await further investigation.

The anatomical location of the antigens, their behavior on extraction in water and strong salt solution, their great instability in the face of various physical treatments, and their susceptibility to incubation with deoxyribonuclease but not with ribonuclease or, under certain conditions, with trypsin, all suggest that they may be deoxyribonuclear proteins.¹ In the mouse these antigens are developed well before birth. It has therefore become necessary to distinguish between antigens responsible for transplantation immunity (these are

nuclear, unstable, and already differentiated in the mouse fetus) and those tissue antigens (cytoplasmic, stable, and not differentiated, in the mouse, until after birth¹⁵) which are known to elicit the formation of circulating serum antibodies capable of agglutinating red cells.^{16, 17} Such a dichotomy is strongly supported by the demonstration that the appearance of circulating antibodies need not coincide with the time of homograft destruction,¹⁸ and that sera rich in hemagglutinating antibodies have the property of *prolonging* rather than curtailing the life of tumor homografts.^{19, 20} The relationship between these two kinds of tissue antigens may be close,²¹ and at present is under investigation.

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ACQUIRED TOLERANCE APPLIED TO EXPERIMENTAL TUMORS

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The fact that several neoplasms can be made to grow in hosts genetically unrelated to the species of tumor origin is not new, and the procedures for successful homotransplantation or heterotransplantation are adequately described in the literature.¹⁻⁵ In the course of the past two years, the writer's work has been concentrated on homotransplantation and heterotransplantation into hosts in which the mechanism of immunity has been impaired by exposure to the tumor antigen *in utero*, that is, at a time when the immunological system is not functioning. The concept of *tolerance* is discussed elsewhere in these pages by Brent and will not be repeated here.

Since most of the work has been recently published,⁶ we will present only a brief summary of data pertaining to the actual homotransplantation and heterotransplantation of 2 mouse and 2 rat tumors, and more space will be devoted to work in progress aimed at the identification of the new or "derived" tumor lines.

TABLE 1 shows the types of tumors used in the present study. These were 2 mouse lymphomas histocompatible with C3H and DBA inbred mouse strains, respectively, and 2 rat tumors grown in the Japanese strain of white rats. Genetic barriers prevent homotransplantation of the 2 mouse lymphomas into mice of other than their own genotype. Although Hauschka *et al.*⁷ maintained the 6C3HED tumor for 84 passages in DBA 2 mice, the neoplasm was not lethal for the DBA mice, and it retained all its original characteristics. The species barrier normally prevents the heterotransplantation of the rat tumors into mice. It will be noted that the 4 tumors employed in this study are of either diploid or hyperdiploid chromosome mode, and thus are not predisposed to survive in a foreign environment.

The techniques used for homotransplantation of these neoplasms into hitherto insusceptible ICR mice followed, in general, methods devised by Billingham, Brent, and Medawar⁸ to make mice tolerant to skin homotransplantation. Attempts to adapt the 6C3HED tumor to ICR mice will serve as an illustration (FIGURE 1). In the first 2 experiments, whole blood of C3H mice or of F₁ hybrids was injected into ICR fetuses which, 6 to 8 weeks after birth, were "challenged" intraperitoneally with the 6C3HED tumor. The tumor grew progressively in all mice, and no difficulty was encountered in establishing these lines on subsequent passages. In the third experiment, actual tumor cells were implanted into fetuses *in utero*. Two of 6 mice born developed subcutaneous lymphomas in the neck region. Suspensions of these tumors injected intraperitoneally into adult ICR mice produced ascites, and passages of ascites fluid resulted in the establishment of the neoplasm in ICR mice. The use of a noninbred stock of mice as recipients can be criticized, but the

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TABLE 1
TYPE OF TUMOR, SIZE OF INOCULUM AND HOST EMPLOYED IN THE STUDY

Original histology	Name of tumor	Chromosome mode	No. of cells in inoculum $\times 10^6$	Mouse or rat strain
Mouse lymphosarcoma	DBA lymphoma	42	12	DBA or DBA \times C3H
	6C3HED	40	6-10	C3H or C3H \times DBA
Rat hepatoma	AH 130	50	*	Japanese rat
Rat sarcoma	Yoshida	40	6-10	Japanese rat

* Impossible to determine accurately because of clumping of cells.

greater resistance of the ICR stock to laparotomy and to fetal inoculation was the reason for the choice.

The adaptation of the 6C3HED tumor was the simplest. Greater difficulties were encountered in the passage of the other 3 tumors in adult ICR mice, even though serial passages through ICR fetuses were made with no trouble. In the case of the DBA lymphoma, 4 serial passages *in utero* preceded the development of ascites in adult ICR mice which could be serially transplanted. The AH 130 hepatoma underwent 2 fetal and 8 intracerebral passages in infant ICR mice before the neoplasm, injected intraperitoneally into adult ICR mice, caused ascites that could then be serially transplanted.

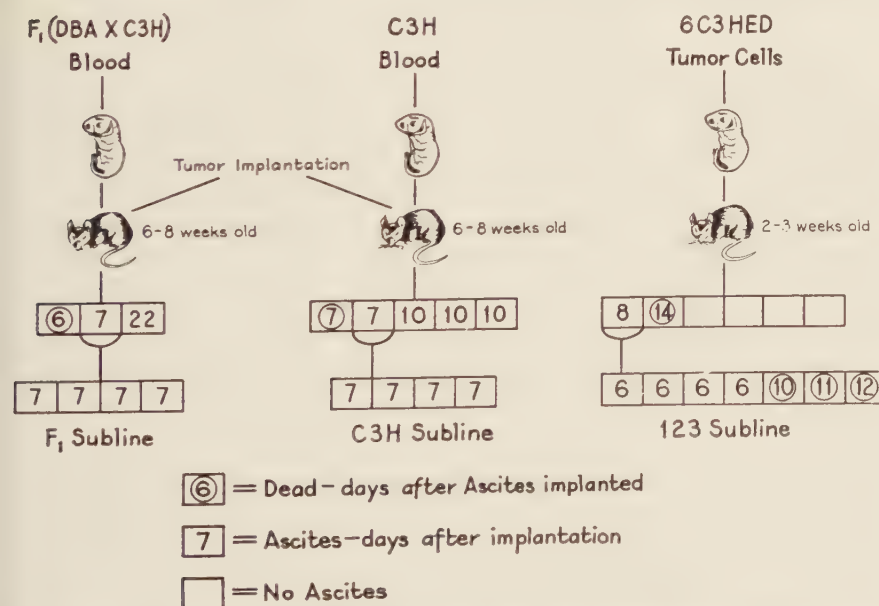


FIGURE 1. The induction of actively acquired tolerance in Swiss mice to 6C3HED ascites tumor.

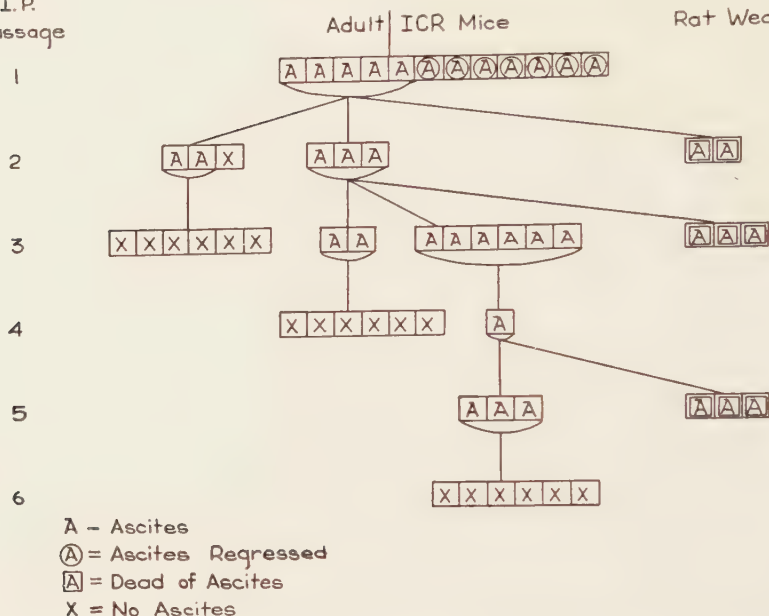
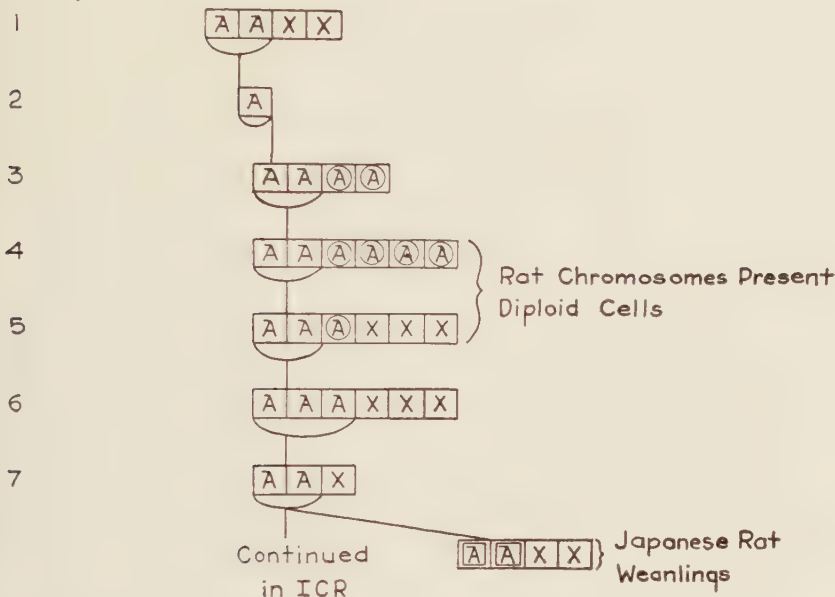
I.P.
PassageCheck in Japanese
Rat Weanlings

FIGURE 2. Unsuccessful attempt to adapt the Yoshida sarcoma rat ascites tumor into ICR mice (8 *in utero* passages plus 33 intracerebral passages in infant mice). Inoculation of a 10 per cent brain suspension from intracerebral passage No. 33.

FIGURES 2, 3, and 4 summarize the attempt to grow the Yoshida sarcoma in ICR mice. In spite of an uninterrupted series of 8 fetal passages, the tumor failed to produce ascites in adult ICR mice. Taking a lead from experience with the AH 130 hepatoma, we implanted the Yoshida tumor into the brain tissue of infant ICR mice. Rapid growth of the tumor resulted; inoculated animals died 5 to 7 days after implantation. In the course of 42 serial passages the tumor retained its original species specificity, as indicated by the death of rats injected intraperitoneally with suspensions of infant mouse brain. Although the same material injected intra-abdominally into adult ICR mice often caused the formation of ascites tumor, transplantation was not possible for more than 1 or 2 generations. The results seemed to be different when inoculum from the forty-second intracerebral passage was used to initiate an intraperitoneal series of passages in adult ICR mice. During the first 7 passages, ascites developed in only some of the mice, and then invariably regressed. The chromosome count of cells representing the fourth and fifth passages indicated diploid modal values, and the chromosomes had the appearance characteristic of rat cells. Rats injected with cells originating from the seventh passage died after developing ascites. Passages of the tumor in ICR mice (FIGURE 4) subsequent to the seventh resulted in a marked change in the properties of the tumor. Mice died after the development of ascites. The chromosome count of cells from the eleventh passage indicated tetraploid modal

I. P. Passage



A = Ascites

X = No Ascites

A = Ascites-Regressed

A = Dead of Ascites

FIGURE 3. The adaptation of the Yoshida sarcoma rat ascites tumor to ICR mice (8 *in utero* passages plus 42 intracerebral passages in infant mice). Inoculation of a 10 per cent suspension from intracerebral passage No. 42 into ICR adult mice.

I. P. Passage

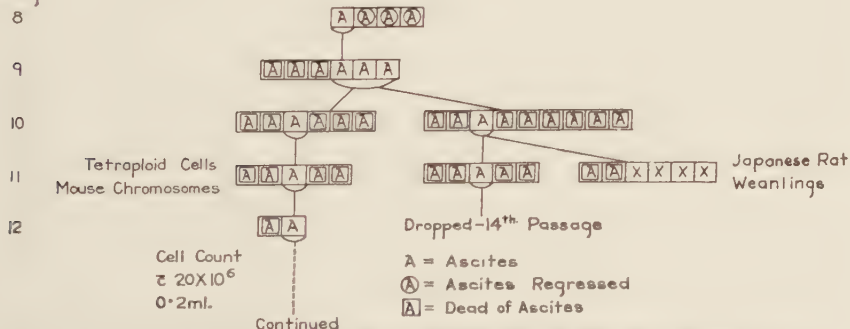


FIGURE 4. The adaptation of the Yoshida sarcoma rat ascites tumor to ICR mice. Inoculation from intraperitoneal passage No. 7 into ICR adult mice.

values, and chromosomes characteristically observed in rat tumors were no longer seen.

Properties of the "Derived" Tumors

The derived tumor lines became indifferent to genetic barriers. TABLE 2 shows the susceptibility of 5 mouse strains, and of one F_1 hybrid strain, to the intraperitoneal implantation of the derived 6C3HED tumor. All strains were almost equally susceptible. However, an inoculum of not less than 1000 tumor cells was required to produce ascites tumor. This is in marked contrast to such ascites tumors as Ehrlich and Krebs, which kill mice after the intraperitoneal implantation of as few as 10 cells. Similar results were obtained with the derived DBA lymphoma.

The susceptibility of rats and of 4 strains of mice to the derived Yoshida tumor at its twentieth mouse passage is shown in TABLE 3. An inoculum of 10 million cells was required to kill Japanese rats; the susceptibility of C3H/He and C57BL/6 mice was not much greater, while small inocula were sufficient to cause tumor development and death of ICR and DBA/2 mice. In contrast, rats were much more susceptible to the intracerebrally passaged (in infant mice) Yoshida tumor (TABLE 4) than were adult ICR mice.

The chromosome number distribution differed widely between the original

TABLE 2
COMPARATIVE SUSCEPTIBILITY OF DIFFERENT STRAINS OF MICE TO THE
SWISS-ADAPTED SUBLINE OF 6C3HED TUMOR

Mouse strain	Cell count of inoculum and mortality ratio of mice			
	4×10^5	4×10^4	4×10^3	4×10^2
C3H/ST	5/5	2/5	2/5	0/5
C3H/He	6/6	1/6	0/6	0/6
A	5/5	0/6	0/6	0/6
DBA/2	5/6	1/6	0/6	0/6
DBA/2 \times C3H/ST	6/6	1/6	0/6	0/6
Swiss	6/6	5/6	0/6	0/6

TABLE 3
COMPARATIVE SUSCEPTIBILITY OF RATS AND DIFFERENT STRAINS OF MICE TO
THE DERIVED YOSHIDA ASCITES TUMOR (TWENTIETH
PASSAGE IN ICR MICE)

Species	Cell count of inoculum and mortality ratio of animals						
	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Japanese rat	3/3	0/3	0/3	0/3			
ICR mice		5/5	5/5	1/5	1/5	0/5	3/5
C3H/He mice		4/5	0/5	0/5	0/5	0/5	0/5
DBA/2 mice		5/5	5/5	3/5	1/5	2/5	1/5
C57/BL6 mice		5/5	1/5	0/5	0/5	0/5	0/5

TABLE 4

COMPARATIVE SUSCEPTIBILITY OF JAPANESE RATS AND ADULT ICR MICE TO THE INTRAPERITONEAL INOCULATION OF YOSHIDA SARCOMA FROM THE FIFTY-THIRD INTRACEREBRAL PASSAGE IN ICR INFANT MICE

Dilution of brain tumor suspension and mortality ratio of animals

Species

	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
Japanese rat	3/3	3/3	3/3	0/3
ICR mice	1/5*	0.5		

* 4×10^6 cells passed into another group of ICR mice with negative results.

and derived lines. The hyperdiploid mode prevailed in cells of the derived lines of the 6C3HED tumor, whereas near-tetraploid values were observed in the derived lines of the other 3 tumors. The cell volume of the derived lines increased appreciably over that of the original tumor, and was accompanied by an increase of from 40 to 100 per cent in cellular deoxyribonucleic acid (DNA) and 100 to 300 per cent in cellular ribonucleic acid (RNA).

The histological appearance of cells of the derived tumors is different from that of the original tumors, and only the occasional observation of island formation by cells of the derived AH 130 tumor suggested their kinship with the original rat tumor.⁶

Attempts at Identification of the Derived Lines

The relative paucity of distinguishing characteristics in the derived lines leads to an inquiry into their identities.

The simplest explanation of their origin and the least flattering to our laboratory would be a mix-up in which the wrong tumor was inoculated into mice: for instance, Ehrlich or Krebs carcinoma instead of the test tumor. In order to exclude this possibility, we shall present some counterarguments that take into consideration factors other than the exactness of supervision of the laboratory work.

The hyperdiploid mode prevailed in cells of all 3 derived lines of the 6C3HED tumor. Since its adaptation to ICR mice occurred on 3 different occasions, it is hardly possible that another tumor (always with hyperdiploid chromosome number distribution) would be "picked up" inadvertently for inoculation 3 times instead of the 6C3HED tumor. As mentioned before, the susceptibility of different mouse strains to the derived lines was low. This relative incompatibility is in contrast to the high degree of susceptibility of the same strains of mice to such ascites tumors as Ehrlich or Krebs. Finally, an irrefutable argument can be offered, based on one characteristic of the derived lines, namely, their susceptibility to the oncolytic effect of viruses. As described before,⁹ the Ehrlich, Krebs, TA3, Sarcoma 37, and MC1M ascites tumors are susceptible to the oncolytic action of Mengo, Bunyamwera, and West Nile viruses, but are resistant to infection with others, among them Semliki Forest

TABLE 5
EFFECT OF INFECTION WITH SEMLIKI FOREST AND WESTERN
EQUINE ENCEPHALOMYELITIS VIRUSES UPON ORIGINAL
AND DERIVED SUBLINES OF TUMORS

Tumor	Semliki Forest virus		Western equine encephalo- myelitis virus	
	Log LD ₅₀ titer of virus*	Oncolysis	Log LD ₅₀ titer of virus*	Oncolysis
6C3HED				
Original.....	2.5	—	<Und.	—
Derived.....	6.0	+	5.0	+
DBA				
Original.....	1.0	—	<Und.	—
Derived.....	5.5	+	6.5	+
AH 130				
Rat.....	<Und.	—	<Und.	—
Mouse.....	4.2	+	6.5	+
Ehrlich.....	1.5			

* In the ascites fluid, 3 to 4 days after inoculation of virus.

and eastern and western encephalomyelitis. This is not the case with the derived lines. As indicated in TABLE 5, the derived lines of the 6C3HED, the DBA, and the AH 130 tumors are susceptible to oncolysis by western equine encephalomyelitis and by Semliki Forest viruses, while the Ehrlich ascites again resisted infection with the latter virus. The effect of eastern equine encephalomyelitis virus seemed to be even more specific, since this virus, as shown in TABLE 6, produced oncolysis only of the AH 130 and DBA derived tumors, without infecting the derived 6C3HED line or either of 2 Ehrlich ascites tumors. The derived Yoshida tumor was effectively destroyed, as shown in TABLE 7, through infection with Mengo, Bunyamwera, West Nile, Semliki Forest, western and eastern equine encephalomyelitis, and vesicular stomatitis

TABLE 6
SUSCEPTIBILITY OF ORIGINAL AND DERIVED ASCITES TUMORS TO THE EFFECT*
OF EASTERN EQUINE ENCEPHALOMYELITIS VIRUS

Tumor	Log LD ₅₀ titer of virus†	Oncolysis
6C3HED		
Original.....	0.5	—
Derived.....	<Und.	—
DBA		
Original.....	<Und.	—
Derived.....	>6.0	+
AH 130		
Rat.....	<Und.	—
Mouse.....	>6.0	+
Ehrlich 4N.....	1.0	—
Ehrlich 2N.....	<Und.	—

* Virus injected intraperitoneally 4 days after implantation of tumor.

† Of ascites fluid.

TABLE 7
SUSCEPTIBILITY OF ORIGINAL AND DERIVED YOSHIDA ASCITES
TUMORS TO INFECTION WITH VIRUSES

Virus	Tumor			
	Original		Derived	
	Log LD ₅₀ titer of virus*	Oncolysis	Log LD ₅₀ titer of virus*	Oncolysis
Mengo.....	1.5	—	8.0	+
Bunyamwera.....	2.5	—	4.5	+
West Nile.....	<0.5	—	1.5	+†
Semliki Forest.....	<2.0	—	>7.5	+
Western equine encephalomyelitis.....	<0.5	—	4.5	+
Eastern equine encephalomyelitis.....	<0.5	—	>6.5	+
Vesicular stomatitis.....	<0.5	—	>7.5	+
Anopheles A.....	<0.5	—	3.5	—
Anopheles B.....	<0.5	—	4.5	±

* Of ascites fluid.

† Experiment in PRI mice. All recovered from tumor.

viruses. The negative results with Anopheles A virus contrast with those obtained with it in Ehrlich ascites.

Exclusion of the mix-up hypothesis does not contribute, however, to a better identification of the derived lines. Has a transformation of the original tumor occurred because of its growth in a tolerant host and subsequent passage to a nontolerant, resistant adult host? Could the tumor become "tolerant" and then become adaptable? Or has the passage of a nonadapted mouse or rat tumor caused the formation of a new mouse tumor? The occurrence of spontaneous tumor must be excluded because of the existence of three 6C3HED "derived" tumor lines that possess similar if not identical characteristics markedly different from those of the other derived lines. It is hoped that future immunological investigations will unravel some of the mysteries.

Until recently, these investigations have consisted of the study of the effect of immunologically activated lymph nodes on derived tumor lines. These lymph nodes were obtained as described by Billingham *et al.*,¹⁰ Mitchison,^{11, 12} and Brncic, Hoecker, and Gasic¹³ from ICR mice following the regression of original 6C3HED or DBA lymphomas. Cells from 12 nodes were injected into mice the day before the implantation of either the original or the derived 6C3HED tumors. Results of 5 such experiments, summarized in TABLE 8, seem to indicate that some immunological relationship, perhaps even specific in character, may exist between the original and derived lines. Yet, as shown in TABLE 9, only negative results were obtained with lymph nodes supposedly activated immunologically by the original DBA lymphoma. Incidentally, this type of work is difficult and quite expensive. More recently, a different approach has been tried in the study of immunological relationships. As described before,⁶ the derived AH 130 line may be "readapted" to Japanese rats, and the tumor may grow for more than 110 serial passages, remaining lethal

TABLE 8

INTRAPERITONEAL PRETREATMENT WITH CELLS OBTAINED FROM LYMPH NODES DRAINING THE REGRESSING 6C3HED ORIGINAL TUMOR IN ICR MICE: ITS EFFECT UPON SUSCEPTIBILITY OF MICE TO ORIGINAL AND DERIVED LINES OF ASCITES TUMORS

Ex- peri- ment No.	Ascites tumor	Strain of mice used	Im- mune lymph cell pre- treat- ment	Survival ratio	Days to death	Probability§
1	6C3HED original	C3H/ST C3H/ST	Yes No	5/5 0/8	0 13, 14, 14, 14, 14, 14, 14, 14	0.001
2	6C3HED derived	ICR ICR	Yes No	2/5 0/5	20, 21, 31 13, 17, 17, 20, 20	0.008
3	6C3HED derived	ICR ICR	Yes No	4/5 0/5	13 18, 18, 21, 21, 21	0.075
4	6C3HED derived	ICR	Yes	0/6	25, 25, 29, 32, 38, 40	
		ICR	Yes*	0/6	18, 21, 21, 25, 27, 27	0.047 0.0039
		ICR	No	0/5	11, 16, 17, 21, 21	
5	6C3HED original	C3H/ST C3H/ST	Yes No†	2/6 0/5	21, 24, 26, 27 15, 15, 16, 17, 19	0.008
	6C3HED derived	ICR ICR	Yes No†	4/4† 0/6	0 24, 24, 27, 27, 29, 33	0.004
	DBA/2 lymphoma original	DBA/2	Yes	0/6	15, 16, 16, 17, 17, 17	
		DBA/2	No†	0/6	15, 15, 15, 16, 16, 17	Not signifi- cant
	DBA/2 lymphoma derived	ICR ICR	Yes No†	0/5 0/6	9, 11, 14, 17, 19 11, 11, 15, 15, 16, 24	Not signifi- cant

* Contralateral—apparently nondraining lymph nodes.

† These mice received equivalent pretreatment with cells derived from normal lymph nodes of ICR mice.

‡ Two mice of this group died 12 and 20 days after tumor implantation without evidence of malignancy.

§ Probability of obtaining such results if treatment produced no effect. Days of death were ranked in order through treated and control groups. The probabilities of the rank totals obtained, under the hypothesis of no difference between treated and control, were found from tables published by Van der Vaart, *Report S 32 (M4) 1950*, from the Statistical Department of the Mathematical Center, Amsterdam, The Netherlands.

TABLE 9

EFFECT OF INTRAPERITONEAL PRETREATMENT WITH DBA LYMPHOMA (ORIGINAL) DRAINING LYMPH NODE CELLS UPON SUSCEPTIBILITY OF DBA MICE TO INJECTION OF ORIGINAL TUMOR

Experiment No.	Pretreatment	Survival ratio	Days to death	Probability
1	Yes No	0/4 0/7	22, 25, 25, 27 12, 15, 16, 18, 18, 18, 18	0.003
2	Yes No	0/5 0/5	18, 18, 19, 21, 21 15, 16, 18, 18, 18	0.008
3	Yes No	0/4 0/5	17, 19, 19, 19 15, 16, 16, 16, 16	0.008

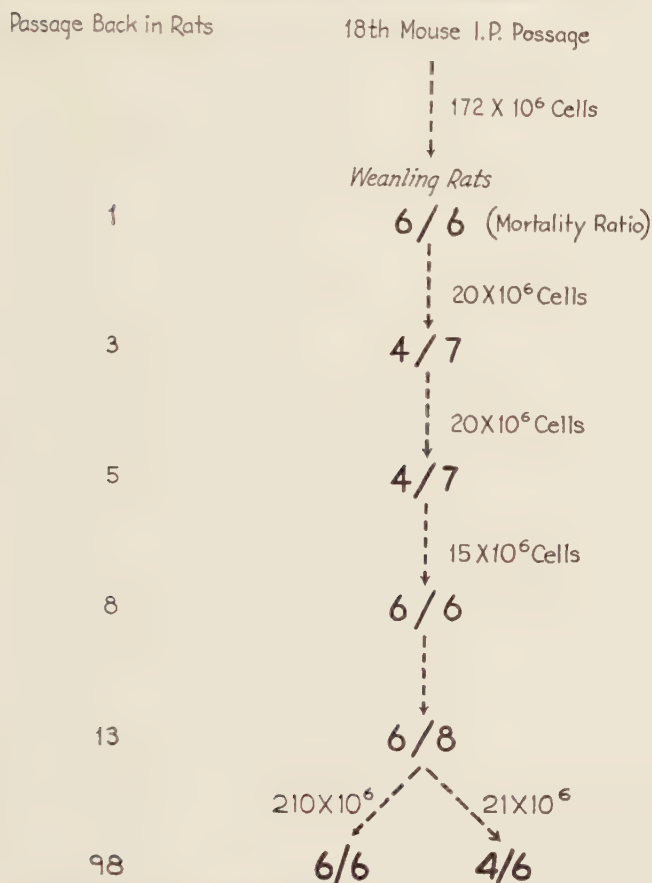


FIGURE 5. Movement of mouse-grown AH 130 rat hepatoma (J. J. Rousseau line).

if injected intraperitoneally in sufficiently large concentrations (FIGURE 5). At present the derived Yoshida sarcoma has also been "readapted" to rats, causing death of the animals in the course of serial passages. It should be mentioned at this point that none of the mouse ascites tumors can be propagated serially in rats, and that they do not cause death of a single animal when directly transplanted from the mouse. The "back-to-rat" passage of the AH 130 tumor, however, seems still to resemble a mouse tumor cytologically. It was injected subcutaneously into 7-day-old Japanese rats (TABLE 10). The subcutaneous tumors regressed, and 21 days later the rats and 2 controls received an intraperitoneal inoculation of the derived, mouse-adapted AH 130. No ascites developed in the rats previously treated with the back-to-rat tumor line, whereas the 2 controls died. Seven days after the booster, the original AH 130 rat tumor was injected intraperitoneally into the previously immunized animals and into 2 controls. The latter died of ascites in 18 days, whereas only 5 of 7 "immunized" rats died, 3 of them after a prolonged incubation period.

TABLE 10
IMMUNOLOGICAL RELATIONSHIP BETWEEN AH 130 DERIVED LINE AND
THE ORIGINAL RAT TUMOR (TESTED IN JAPANESE RATS)

Original inoculum	Subsequent inocula	Days between inocula- tions	Ratio of rats developing ascites	
			Test animals	Controls
"Back-to-rat" passage 89	(1) AH 130 (71st ICR) (2) AH 130 original	21 7	0/7 5/7 (12, 14, 23, 23, 27)	2/2 (3, 3)* 2/2 (18, 18)*

* Figures in parentheses = days to death.

TABLE 11
IMMUNOLOGICAL RELATIONSHIP BETWEEN DBA DERIVED AND
ORIGINAL TUMORS (TESTED IN PRI MICE)

Original inoculum	Subsequent inocula	Days between inocula- tions	Ratio of test mice		Ratio of control mice	
			Developing tumor	Dead of tumor	Devel- oping tumor	Dead of tumor
DBA derived	(1) West Nile virus	7	11/11	0/11*	1/1	1/1
	(2) DBA derived	14	0/6	0/6	5/5	5/5
	(3) DBA original	14	0/3	0/3	3/3	0/3

* Following infection with West Nile virus 7 days after tumor implant.

Another variant of the same type of approach now under investigation is the implantation of the derived lines in PRI mice, and the subsequent destruction of the tumor at the peak of its growth by West Nile virus, which does not affect PRI mice. Following booster inoculations with the same tumor, mice previously immunized have been found to be resistant to the original tumor. An example is given in TABLE 11. PRI mice were inoculated with the DBA-derived tumor and infected with West Nile virus 7 days later. Fourteen days after virus treatment, a booster injection of the derived line was given, and 14 days after that, DBA original tumor was implanted subcutaneously into 3 of the mice and into 3 controls. No growth was observed in the mice previously treated with the DBA-derived line. In contrast, the tumor grew in controls, but ultimately regressed because of incompatibility with the host. These experiments are still in the preliminary stage, and it is yet unknown if the results will make possible the identification of the derived tumors.

It is quite obvious that classic immunology, immunogenetics, and genetics are essential disciplines that must be employed in the follow-up of an originally innocent attempt to make resistant mice tolerant to tumors and, possibly, to make tumors tolerant to a sojourn in foreign territory.

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HETEROTRANSPLANTATION OF TUMORS*

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Heterotransplantability is a developmental acquisition and not an attribute of cancer from its inception. The development of the property is of vital concern to the bearer, inasmuch as it coincides with the attainment of the ability to metastasize, but it is also of great significance from the point of view of biology as a manifestation of independence of the factors governing the interspecies transfer of tissue. The fully developed cancer, free of contaminating normal donor tissues, survives transfer to alien species, invokes a blood supply and, in actuality, becomes a part of the new host. In contrast, the heterologous transfer of adult tissues is associated with an inflammatory foreign-body response, segregating and destroying the graft, and a comparable reaction follows the transplantation of neoplastic tissue in its premetastazable phase.

The factors determining the highly distinctive transplantation reactions of cancer have been the subject of continued study; the object of this report is to discuss present findings as they relate to four questions. First, is heterotransplantability an essential and general attribute of all cancer? Second, is the property always associated with metastasizability? Third, is the property limited to cancer or is it shared by other tissue states? Fourth, what is the fundamental nature of the change permitting heterotransplantability?

Exploration of the first question consisted of a general survey of the cancers of lower animals and of man with respect to heterotransplantability. The tumors tested included a wide variety of morphologic types derived either from transplanted or spontaneous growths in rabbits, mice, rats, hamsters, dogs, and man. Representative fragments of the tumors were transferred to a number of different species, utilizing a diversity of transplantation sites; in no case was the tissue or the recipient animal subjected to special treatment. In brief, it was found that, irrespective of the species of origin, all tumors capable of metastasis possessed the ability to grow on heterologous transfer.¹⁻⁶

9-17, 19-24, 26, 29, 31, 32

Occasional tumors distinguished by a minimum of stroma or dermoplastic reaction, such as the Brown-Pearce and V-2 rabbit carcinomas, grew immediately on subcutaneous transfer to dba mice or hamsters. However, in the vast majority of cases, transfer from the foreign species was successful only when the brain or the eye was used as a transplantation site. In such instances, second-generation transfers within the new species usually could be effected in other bodily regions, and several heterologous tumors have been maintained for long periods of time by serial passage in the testicle or subcutaneous space.

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Serial transfer in the alien species is not accompanied by notable changes in morphology or behavior. However, return of the growth to the parent species is usually associated with more rapid growth than is characteristic of the same tumor maintained by continued homologous transfer.

The incidence of "takes" in the first transplanted generation varied widely, and two major factors were found to be operative in this respect. One concerned anterior-chamber transplantation and was related to the species of the donor and recipient. It was found that a grouping of species with reference to the ability to synthesize vitamin C coincided with susceptibility or resistance to heterologous eye transfer. Transfer between species with the same type of C metabolism (man and guinea pig) was comparatively easy, while transfer between species with different types (man and mouse) was difficult. The possibility that this relationship might be a true reflection of vitamin C metabolism rather than merely coincidental is enhanced by the fact that, while such considerations pertain to the eye and other bodily regions, they do not apply to the brain. Human cancers grow as readily in the brain of the mouse as in the brain of the guinea pig, whereas transfer of such tissue to the mouse eye is rarely successful. Similarly, the Brown-Pearce rabbit tumor fails to survive transfer to the guinea pig eye, yet grows well in the brain of this species. It is suggested that the anomalous states of the brain in relation to vitamin C may form the basis for such behavior. In any case, it would appear from transplantation reactions that the brain substances of different animal species bear a closer relationship to each other than do other bodily tissues.

Another factor determining the incidence of takes concerns the tissue used for transfer, and selection of proper fragments is by far the most important step in the procedure. In addition to essential parenchyma, all cancers contain stroma and many are infiltrated with dermoplastic connective tissue. This tissue is adult in nature and will not grow on transfer. In fact, its presence in quantity will give rise to a foreign-body reaction in the alien host and result in death of the transplant. It is essential, therefore, that selection be based on the content of tumor parenchyma, and some knowledge of gross pathology is necessary for such a differentiation. Frozen sections obtained from different areas of the tumor mass are of considerable aid in some cases. It is obvious that necrotic tumor or normal tissue adjacent to the tumor is not material suitable for transfer.

Transplantation experiments involving different mouse strains indicate that the stroma of the transplanted tissue may also influence the success of transfer. A number of tumors growing in a C_3H strain different from the C_3H strain of origin were transplanted subcutaneously to DBA, C57, Bagg albino, and A albino mice and to the eyes of guinea pigs. Subsequently, the experiment was repeated, utilizing the same tumors growing in DBA mice and in guinea pigs. The incidence of takes in the experiments showed a wide variation related to the strain or species of the donor, being 13.4 per cent with inocula derived from the C_3H strain, 47.7 per cent with tissue derived from the same tumors residing in DBA mice, and 71.4 per cent when the inocula consisted of the mouse tumor tissue growing in guinea pig eyes.¹² Inasmuch as the parenchyma of the tumor persists on transfer and the stroma is a prod-

uct of the new host, it would appear that the primary difference in the tissues used was in stromal composition. It is conceivable that the connective tissue of C₃H and DBA mice and guinea pigs, comprising the stroma of their tumors, may differ in the ability to induce foreign-body reactions when introduced into foreign hosts. In such cases, the fate of the parenchyma would be determined to a large extent by the intensity of the ensuing inflammatory reaction, and survival and growth or death and destruction would depend on the relative compatability of the connective tissue of the donor and recipient.

While such factors may be of influence in determining the incidence of takes, by far the most important element in the success or failure of heterologous transfer is the technique employed. Also, it should be emphasized that the haphazard methods utilized in many laboratories reporting negative results in attempts to transfer human cancer to laboratory animals constitute the primary cause of failures. In some such cases the failure to obtain takes has been attributed to the operation of immunological laws that prohibit the interspecies transfer of tissues, and for this reason it seems cogent, in the present context, to repeat the more important details of the anterior-chamber procedure.

The animal is immobilized with tie strings on an operating board in such a manner that the desired eye is presented to the operator. The cornea is anesthetized by contact with a 5 per cent aqueous solution of cocaine administered with a medicine dropper. An incision is made at approximately the midpoint of the upper border of the limbus with a sharp, double-edged corneal knife. The knife is directed slightly forward so that the blade enters the anterior chamber without damage to the iris. The pressure necessary to pierce the cornea is sufficient, even with a sharp knife, to rotate the eyeball beneath the lower lid and conceal the operative field. A short, quick thrust of the knife, however, results in an adequate opening and the temporary obscurity is inconsequential. Withdrawal of the knife is accompanied by the escape of a small amount of aqueous humor but, unless the iris has been cut by a misdirected knife, there is no bleeding.

Transfer of the tumor is effected by means of a trocar commensurate in diameter with the corneal incision. The trocar should be equipped with a tight-fitting plunger and a short beveled mouth with all edges filed to smoothness. A small fragment of tumor is placed in the mouth of this trocar and forced into the barrel, a step greatly facilitated by withdrawing the plunger to produce suction. The tip of the trocar is inserted through the incision a short distance so that the bore is entirely within the chamber and the fragment is expelled. It is important that all manual pressure about the eye and the head of the animal be released before withdrawing the trocar to prevent escape of the fragment. Finally, the fragment is forced into the inferior angle of the iris by applying light pressure along the corneal surface with a blunt instrument. The incision is not covered.

The procedure is executed with rapidity, and the technique is extremely simple, yet several points require further emphasis. The corneal knife should be sharp and of such width that a stab wound will admit the trocar. The use of a narrow knife necessitates side cutting to obtain a sufficient opening

and this may be attended by iris damage with consequent bleeding. Pointed surgical blades or single-edged corneal knives are undesirable, for the triangular cut produced heals slowly and may result in herniation of the iris.

The fragments of tissue to be transplanted should be cut from the tumor mass with sharp instruments and should not exceed 1 mm. in diameter. The placing of the fragment in a wedged position in the inferior angle of the iris is important for early vascularization. Occasionally, fragments so placed work loose shortly after operation, and an examination with required readjustment is desirable before returning the animal to the colony. Fixation occurs within a few hours, and further check is unnecessary.

The tumor tissue may be transplanted immediately after removal or stored at icebox temperature for several days. In the latter case, it should be kept free of contact with hospital physiological saline or other noxious fluids. It is essential that the material be free of infection and that surgical sterility be maintained throughout all manipulations.

The technique as outlined, with minor modifications for special sites such as the brain, was used in the test experiments cited above, and it resulted in the successful heterologous transplantation of a wide variety of cancers obtained from many animal species. Accordingly, it was concluded that heterotransplantability is a general and essential property of cancer—if cancer is defined as a metastasizable growth. It should be noted that, although the lymphomas are usually classified as sarcomas, they do not belong to the heterotransplantable group of tumors. Many attempts have been made to transfer the different varieties occurring in man, but no evidence of a take has been obtained thus far. Several hamster lymphomas and a large number of mouse tumors so classified have been extensively tested, but in only a single instance has transfer been successful. This instance concerned transplantation of the Gardner lymphoma to the subcutaneous space of hamsters. Takes occurred in approximately one out of every thirty hamsters used, and the incidence did not increase with continued serial passage.

No constant morphologic feature distinguished the many tumors found to be capable of heterotransplantability in the study described, but there did appear to be a consistent association of this property with metastasizability. The relationship has been under continued investigation, and some of the findings are of interest in the present context.

Early experiments were carried out with the spontaneous tumors of laboratory animals. Biopsies were performed at short intervals throughout the course of the growths, and the tissues obtained were transplanted autologously, homologously, and heterologously. The status of the disease in relation to metastasis was determined by complete autopsy of representative animals at various phases of tumor development. In brief, it was found that premetastasizable lesions could not be transplanted to unrelated animals of the same species, whereas growth was readily obtained during later metastasizable stages of the same tumors. For example, biopsy fragments of a rabbit breast papilloma were easily transplanted elsewhere in the same host or to other rabbits bearing breast papillomas, but they failed to survive transfer to normal rabbits or to alien species. In contrast, at a later stage of development when

the papillary growth had attained the ability to metastasize, transfer to normal animals irrespective of species was readily effected.

An identical situation was found to characterize the transplantation reactions of all of the spontaneous tumors studied, including a large series in rabbits and mice and a smaller group in rats and hamsters. From initiation to the attainment of the ability to metastasize, the growths are biologically dependent in the sense that special factors are required for their continued existence. They can be transplanted back elsewhere in the primary host or to other animals bearing spontaneous tumors of the same type, but they do not survive transfer to normal unrelated animals. In other words, they are dependent for their continued existence on factors peculiar to the tumor-bearing animals, and such factors are not supplied by normal animals. With the attainment of the ability to metastasize, the tumor becomes independent of these factors, or autonomous, and will grow in their absence.

Comparable phases of dependency and autonomy also distinguish the developmental course of human tumors. A similar investigation with autologous and homologous transfer is obviously impossible; however, considerable information can be obtained from the heterotransplantation of successive biopsy specimens secured at intervals throughout the period of development. Several experiments of this type have been completed, and the results indicate a complete parallelism with the tumors of laboratory animals.¹⁸⁻¹⁹ That is, the human tumors were not transplantable to the guinea pig at early stages of development, but became transplantable during their later stages. Further evidence of the analogy between human and animal tumors can be derived from the results of a transplantation study utilizing routine operative specimens. In a large series, it was found that patients with heterotransplantable tumors invariably died within six months of operation, while a large proportion of those with nontransplantable tumors have survived for many years. The rapidly fatal course in the first instance suggests that metastases were present at the time the operative specimen was obtained, while the survival of the latter group is certain evidence that metastasis had not occurred at the time of transfer.

As a result of a long search, two exceptions have been found to the generalization that only metastasizable tumors are heterotransplantable. One concerns the human brain tumors: the glioblastoma multiforme and the medulloblastoma. Neither of these malignant tumors ever metastasizes outside of the brain, yet both are transplantable to guinea pigs during their late stages. It is significant that, unlike other human tumors that survive transfer to other bodily regions after residence in the pig's eye or brain, the glioblastoma and the medulloblastoma are capable of growth only in the eye or brain. It is suggested that these tumors, being anaplastic brain cells, possess to an even lesser degree the poor stroma-evoking properties of brain cells. Their qualities in this respect are sufficient to evoke stroma from the high responsive connective tissues of the eye and brain, but are insufficient to stimulate a response of connective tissue in other regions adequate to support and nourish either disseminated or transplanted tumor cells.

The second exception concerns the virus-induced tumors: the Shope papil-

oma and the Rous sarcoma. Both of these tumors possess the ability to survive and grow in heterologous species from their earliest stages of development.²¹⁻²⁵ Transplantability is present from inception, yet metastasizability is acquired only after an extended developmental course. Investigation suggests that the intracellular virus is analogous in its operation to the constitutional factors concerned in the dependence of other tumors and that the transplantability and growth of the Shope and the Rous in normal animals is conditioned on the concomitant transfer of their dependent factors. It has been found, for example, that regressing, presumably virus-free papillomas in domestic rabbits will not survive subcutaneous transfer to the hamster. However, if fragments of such a papilloma are reinfected with wild virus, transfer to the hamster or other alien host is readily effected.

A conception of the virus as a conditioning factor analogous in its action to the constitutional factors concerned in the dependence of other tumors and located in the cell rather than in the host implies that transfer of an infected cell would necessarily be accompanied by transfer of the factor on which its growth depends. Thus, the special environmental conditions requisite for the growth of other tumors transplanted during dependent phases of development are replaced by intracellular components in the case of virus tumors and, in this respect, the survival and growth of transplants are independent of the constitutional status of the host. The final epidermoid carcinoma-metastasizable stage of the Shope papilloma is characterized by the absence of detectable virus, and the ability of the tumor to survive heterologous transfer at this period of development is evidence of the attainment of autonomy or independence of conditioning factors such as distinguishes the metastasizable phases of other nonvirus tumors.

In a further attempt to determine the general relationships of heterotransplantability, the experiments were extended to include tissue states other than neoplastic. The more important of the states studied were hyperplastic, adult, and embryonic. With two exceptions, hyperplastic tissues and all of the normal adult tissues failed to survive in alien hosts. The exceptions consisted of pia-arachnoid and Schwann's sheath and, although both of these tissues persisted for many months with vascularization and complete preservation of architecture, they showed no observable increase in size. In contrast, embryonic tissues obtained during the first half of gestation survived and increased in size in the eye or brain of the alien host.⁶ Moreover, heterologous transfer could often be effected successfully with tissue transferred immediately from the embryo to the subcutaneous space.

In brief summary, it seems probable from a comprehensive sampling of different animal species and of a wide variety of morphologic types that heterotransplantability is a general and essential property of cancer. The property is a developmental acquisition and not an attribute of cancer from its inception. It is associated with the attainment of metastasizability; prior to this event, the tumor is dependent for its continued existence on conditioning factors peculiar to the tumor-bearing individual and not supplied by normal individuals. In the case of the virus-induced tumors, the dependent factor is an intracellular virus, and its transfer to the new host is concomitant with transfer

of the cells of the tumor. However, with the development of metastasizability, the tumors become independent of the virus-conditioning factor and survive heterologous transplantation in its absence. The property of heterotransplantability is not limited to cancer tissue, but is shared by embryonic tissue during the first half of gestation.

The central problem in the investigation reviewed in this report concerned the nature of cellular changes resulting in the ability of cancer to survive transplantation to alien hosts. A successful transfer of this type is in sharp contrast to the reaction encountered following the heterologous transplantation of other tissues of adult individuals and, presumably, it is associated with alterations in intrinsic character that may be basic to the cancer cell. Some of the findings noted constitute suggestive leads, and pertinent exploratory experiments have been undertaken.

It has been suggested that the success of heterologous transfer depends on the use of the brain and eye as transplantation sites and that it relates to the existence of a barrier preventing the passage of antibody between the blood and these organs. However, in experiments involving the eye, it was found that, following tumor growth in the anterior chamber, both the testicle and the anterior chamber of the opposite side were resistant to reinoculation.⁷ On this basis, it was concluded that the aqueous humor was not an isolated fluid, but that it participated in general body reactions and that its susceptibility to tumor transfer was not determined by an antibody barrier. In any case, it should be emphasized that, in both the eye and the brain, transplanted cancer fragments rapidly become vascularized and, in actuality, are parts of the new host rather than tissue cultures growing in fluid-filled compartments.

It seems significant that the second-generation passage of cancer within the alien species can be effected in general bodily regions, whereas the first passage is usually successful only in the eye or brain. The tissues used for transfer in such cases differ with respect to at least one component. The fragments employed in the primary heterologous transfer consist of both parenchymal cancer cells and a stroma made up of normal connective tissue of the donor species. Transplantation to the new host is associated with death of the stromal component, and the persistent parenchyma is supplied with a new connective-tissue scaffolding and vascular supply derived from the foreign species. Thus, the species' identity of the stroma pervading the cancer tissue used in transfers outside of the brain or eye appears to be a factor determining the fate of the transplant; homologous stroma is associated with survival, and heterologous stroma with an inflammatory reaction. Actually many cancers, particularly those of human origin, also contain a variable amount of desmoplastic tissue, representing a reactive proliferation, and such tissue necessarily contaminates fragments used for transfer and contributes to the inflammatory response. A desmoplastic reaction does not occur in the eye or brain; the resulting growths are usually medullary, and stroma is present only in small amounts. Extreme examples of the latter are the Brown-Pearce and V-2 rabbit tumors; as previously noted, transfer from the rabbit's eye to the subcutaneous space of hamster or mice is accompanied by a minimal inflammatory reaction and a high incidence of takes. Other tumors, more

organized and with a higher content of stroma, require a passage through the eye or brain of the foreign species before subcutaneous transfer becomes possible.

The point to be noted is that factors resident in extraneous adult tissues rather than in the cancer cells themselves appear to be the basis of the foreign-body reaction accompanying transfer to bodily regions of alien hosts. In such a case, a question arises as to why such factors are not operative in the anterior chamber and the brain. In fact, however, they are operative, as evidenced by the absence of takes subsequent to the transfer of cancer highly contaminated by adult tissues. The superiority of the eye and the brain as transplantation sites stems, not from a complete insensitivity to foreign bodies, but rather from a lessened sensitivity, so that minor degrees of contamination are tolerated. This circumstance is illustrated by transfer of comparable human cancer fragments, selected by frozen section for high parenchyma content, to the subcutaneous space and eyes of guinea pigs. An almost immediate foreign-body reaction occurs in the subcutaneous space, so that within two days the transplant is encapsulated or destroyed. In contrast, the fragment in the anterior chamber excites no reaction and, except for a slight increase in size, remains unchanged in appearance for a period of three to four days. Sections taken after this interval show an increase in the proportion of parenchyma over that present in the transplanted fragment, and there is no evidence of an inflammatory reaction. It seems probable that the parenchyma of the fragment, being the preponderant intermitotic element present, continues to proliferate after transfer, imbibing nutrient from the surrounding aqueous humor, whereas the included dermoplastic connective tissues, being differentiated and postmitotic, remain unchanged or actually diminish in amount. Stroma does not increase and, on section, the new parenchyma resembles the stromaless growth of a tissue culture. Thus, the stromal-parenchymal relationships are altered prior to the occurrence of a host reaction; when this occurs, the content of connective tissue is comparatively less than that which characterized the fragment introduced into the subcutaneous space. In any case, the reaction is different in character and shows none of the acute exudative features found in other bodily regions. It is primarily proliferative and results in stromatization rather than encapsulation of the transplant.

The absence of factors capable of inducing a foreign-body reaction in alien species would appear to be a special attribute of cancer cells, and there is much evidence to suggest that the deficiency relates to the antigenic properties distinguishing the normal cells of different species. Human cancer cells freed of human connective tissue by anterior-chamber passage in a guinea pig survive and continue to grow in the presence of antibody against human tissue, and second-generation passage of human cancer is effected as readily in pigs immunized against human tissues as in normal, untreated pigs.

Watson,³⁵ working in this laboratory, investigated the specificity of heterologous tumor transplants by means of a specially devised complement-fixation reaction utilizing chicken complement. She found that antigens prepared from first generation transplants in alien hosts failed to react with any of the antisera to the original host species. Extracts of tumor grown in guinea pigs,

regardless of the species of origin, fixed complement only in the presence of anti-guinea pig sera. On the other hand, when the same tumor was transplanted from the guinea pig to the rabbit, it failed to react with antisera of either the species of origin or the guinea pig. In brief, the same alien tumor transplanted to a variety of foreign species manifested only the antigenic properties of the tissues of the species in which it was resident at the time of test. Inasmuch as the connective-tissue content of the tumor changed to that of the new species following transfer, and as the species specificity of the transplant varied accordingly, the reactions noted above can be attributed to its stromal component. However, the parenchyma of the transplant is not a product of the new hosts, but persists on transfer, and its failure to react with any of the antisera to the original host species is a significant finding.

In a continuation of this work, Maculla³⁰ separated the saline-soluble cytoplasmic components and nucleoproteins of heterotransplants and studied their species-specific characteristics. By means of rigidly standardized complement-fixation procedures, the cytoplasmic components were found to have undergone complete alteration, and they appeared, as in the previous investigation, to be under the direct influence of the host supporting the growth of the transplant. This host influence was confirmed by returning the tumor to the species of origin when it was found to possess again the cytoplasmic components characteristic of that species. Alterations in the caliber of the reactions of the antisera to nucleoproteins and residues obtained after repeated saline extractions suggested that the nucleus had also undergone chemical changes following heterologous growth.

The mechanism whereby the cancer cell attains the unique property of responding to the influence of the foreign host by altering the chemical constitution of its cytoplasmic components to correspond with that of the new species has been under continued study. One of the more suggestive findings stems from a study of developmental changes throughout the Shope papilloma-carcinoma sequence. In brief, the pertinent experiment consisted of the *in vitro* infection of normal adult rabbit skin, papilloma tissue, and epidermoid carcinoma tissue with cottontail virus; transplantation to the brains of rabbits immune to the virus; and a test of the resulting growths for the presence of infectious virus.

Despite residence in the immune environment for a month, both the papilloma derived from the skin grafts and the transplanted papilloma tissue were found to contain infectious virus. In sharp contrast, infectious virus could not be demonstrated in transplants of the epidermoid carcinoma, although a high titer was present in control transplants to normal, nonimmune animals.²⁸ Inasmuch as the virus is intracellular in location, the implication is that immune bodies failed to gain entrance into the cells of either the adult skin or the papillomas, but did gain entrance into the cells of the carcinoma.

Additional experiments were designed to test the suggestion that the development of autonomy is associated with an increased ability of the cell to take up protein from the plasma. Radioactive glycine was incorporated into serum protein and injected into rats bearing the Walker sarcoma, and the uptake

by the tumor was found to be $2^{1.2}$ to 10 times that of other tissues.^{2, 3} Again, following the injection of antibody against bacteriophage into hamsters bearing a malignant melanoma, extracts of the tumor and of various organs were plated with phage and bacteria. The high colony counts in plates containing tumor extracts, in contrast to the low counts in plates containing extracts of other tissues, demonstrated their differential antibody content.³³

The increased permeability of the cancer cell to plasma proteins is of interest as a possible mechanism to account for the absence of virus in the final epidermoid carcinoma stage of the Shope papilloma, but the alteration is not limited to this particular tumor and is of pertinence to other problems related to the biological behavior of cancer. In the present context it has a bearing of possible significance on the observed alteration in the cytoplasmic constitution of cancer cells incident to heterologous transfer. It is suggested that the increased permeability of the cells permits the passage of protein characteristic of the new species through the cell membrane and that the alteration in species specificity is consequent to its incorporation in cytoplasmic components. Such an alteration occurring in transplanted cancer cells during the tissue-culture phase of growth in the eye or brain would result in a cellular mass with the species characteristics of the recipient host, and the ensuing reactions would not be different from those following the transfer of homologous cancer.

A comparable situation may exist with respect to the heterologous transplantation of embryonic tissues. Early embryonic tissues survive heterologous transfer, whereas late developmental stages behave like adult tissues and induce a foreign-body reaction. Transfer to bodily regions other than the eye is successfully effected with much greater frequency than are similar transfers of cancer tissue. The basis of this variation may be the absence of adult stroma and desmoplastic connective tissue, but whether or not the factors determining the heterotransplantability of embryonic cells are of the same nature as those concerned in the heterotransplantability of cancer cells has not been resolved and pertinent investigation is in progress.

The antigenicity of early embryonic organs and tissues has been the subject of comparatively little study. Burke and others¹ have found evidence of progressively changing antigenic specificity during development. Maculla³⁰ noted that, while the antigenic components of certain organs such as the liver and lung were similar in their embryonic counterparts, the antigenic components of adult and embryonic states of other organs such as the kidney and spleen were immunologically distinct. Accordingly, the heterotransplantability of early embryonic tissues may relate to the absence of species specificity and the loss of this ability in later stages to the development of antigenicity.

The status of the early embryonic cell with reference to permeability has not been determined. The permeability of the cancer cell is not shared by embryonic cells during the second half of gestation, for embryonic mouse lung of this age, infected *in vitro* with influenza virus and grown in immune adult mice for a week, contains an increased titer of virus. Mouse cancer tissue so treated and transplanted to a different site in the same immune

animal, for the same period of time, contains no demonstrable virus.²⁴ It would appear, therefore, that cancer cells and late embryonic cells differ in their permeability to antibody.

Similar experiments utilizing early, heterotransplantable embryonic tissue have not been performed. However, it appears significant that such tissue differentiates and matures into morphologically adult structures in the alien host and persists as such without foreign-body reaction. The absence of an inflammatory reaction following transfer is evidence that, at that time, the embryonic tissue did not possess the antigenic characteristics of the parent species, and its subsequent failure to occur implies that differentiation and maturation were not associated with the development of these qualities. Inasmuch as antigenic qualities would have developed with differentiation and maturation had the embryonic tissue remained in its natural host, it seems clear that the heterologous host exerted a directive influence. It is of interest in passing to note that, although the antigenic characteristics of the parent species are lacking in mature embryonic transplants residing in alien species, the specific disease susceptibilities of the parent species are retained. Thus, if transplants of embryonic rabbit skin are removed from the subcutaneous space of the hamster or the brain of the mouse after long residence and infected with the Shope papilloma virus prior to retransfer to the alien species, typical papillomas arise in the transplants.²⁷ Apparently, the species factors determining susceptibility are different from those determining antigenicity, and the expression of the latter factors depends on the nurture rather than the nature of the tissue.

The experiments reviewed in this report demonstrate that cancer is not a sudden transformation of normal cells but, on the contrary, represents a final step in a developmental process. Throughout the process, the tumor may remain static morphologically, but it does undergo profound biological changes. The most dramatic of these occurs near the end of its course, and consists of the attainment of the ability to metastasize. Coincidentally, the tumor attains the ability to grow on heterologous transfer. Investigation suggests that the latter property stems from a modification in the cell wall leading to an increase in permeability to proteins. Characteristic proteins of the alien species are incorporated into the cytoplasmic components during the early phases of transfer, and the cells fail to excite the inflammatory reaction typical of transplants possessing the antigenic qualities of the donor species.

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IMMUNOLOGICAL RESPONSES ELICITED IN THE HETEROLOGOUS HOST BY TRANSPLANTABLE HUMAN TUMORS: USE OF THE CONDITIONED RAT AS A TEST MEDIUM*

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The problem of the role of humoral and cellular "antibodies" engendered in the immunological reaction of animal hosts to tumors and grafted tissues has produced a voluminous literature that has been reviewed elsewhere.¹ In general it has been concluded that the production of humoral cytotoxins is a primary manifestation of immunized heterologous hosts, although such cytotoxins have been variable, weak, or difficult to demonstrate in the sera of animals treated with homologous tissues.² In contrast to the latter finding, the importance of the "toxins" associated with lymphoid cells in homologous immunological reactions has come recently into sharp focus and has been emphasized through the ingenious methods devised by several investigators: notably Algire and his associates,^{3, 4} who have used an intraperitoneally implanted diffusion chamber containing cells to be tested; Mitchison,^{5, 6} who has employed passive transfer techniques similar to those of Chase;⁷ and Billingham, Brent, and Medawar⁸ who have injected mice *in utero*, thus causing an "immunological paralysis" of their lymphoid tissues and producing a tolerance in these animals toward the tissues of the species used in immunization.

The possible relative values and potency of the humoral as against the cellular cytotoxins produced in immunized homologous or heterologous hosts have not been determined as yet. One reason for this is the lack of a good test method. Tissue cultures, which at first seemed so promising for this purpose due to the enthusiastic, if conflicting, reports of Lumsden,^{9, 10} of Niven,¹¹ and of Phelps,¹² have proved disappointing, as the findings of Harris¹³ and of Medawar¹⁴ attest. The more usual bacteriological procedures involving agglutination, precipitation, and complement fixation are not equally applicable to the two types of material (sera and cells) under investigation. In the experiments to be reported herein, use has been made of the X-irradiated and cortisone-treated animal^{15, 16} as a "neutral" *in vivo* test medium for the determination of the amount of damage incurred by tumor cells that had been treated with test sera and cells prior to implantation of these hosts. All the studies were concerned with the immunological responses of a heterologous species (the rat) to implanted human tumors. Both humoral and cellular cytotoxins were detected, and their relative potency and length of duration have been estimated.

Materials and Methods

The animals, materials, and many of the methods employed for these experiments have been described in detail elsewhere.^{15, 17, 18} Rats used for immuniza-

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tion were usually young adult Wistar-strain females, approximately 200 to 250 gm. in weight, although a few were of weanling age and size (50 to 60 gm.). X-irradiated rats employed as a test medium were also Wistar females, all of weanling age, that had received 150 r total-body X irradiation 1 to 3 days prior to implantation. The 3 transplantable human tumors used for immunization were human epidermoid carcinoma No. 3 (HEp 3), human sarcoma No. 1 (HS 1), or human epidermoid carcinoma No. 4 (HEp 4). These were removed under aseptic conditions from their conditioned rat hosts, minced with scalpels in a buffered Locke-Ringer's solution, and injected subcutaneously through a No. 18 needle into a flank of the normal rats to be immunized and also into a few conditioned hosts. The usual implantation was 1 ml. of a suspension containing 0.5 gm. of tumor before chopping. None of the injections developed into palpable nodules in normal hosts, although they invariably grew well in the control, X-irradiated and cortisone-treated rats. The disintegrated tumor tissue, also used for immunization, was first prepared by mincing freshly removed tumor without added fluid. Five gm. of the neoplastic tissue was then suspended in 30 ml. of buffered Locke-Ringer's solution and placed in a 9-kc. Raytheon sonic oscillator for 1 hour. After such treatment of the tumor cells no nuclei or recognizable cell fragments remained, as far as could be discerned with the ordinary microscope, nor did such material ever produce growth in properly conditioned test hosts. Normal rats were given 3 ml. each of the suspension. This amount equaled 0.5 gm. of original tumor per rat, or the same quantity used for immunization of the animals that received minced and fresh whole tumor.

The Locke-Ringer's solution that was used as the suspending medium for all cells had been buffered to a pH of 7.2 with a McIlvaine buffer on the day of the experiment and contained 6 mg. of added glucose per ml. It was designated as BGR (buffered-glucose Ringer's solution).

HEp 3 was the test tumor employed. It was prepared in two ways: either by especially fine mincing in the manner already described, or by pressing it through a 40-mesh Monel metal sieve with a pestle, and then sieving this suspension again through a fine piece of sterilized silk by means of pressure applied with a large syringe. The original weight of tumor used for either procedure was 300 mg. per 1.5 ml. of buffered Locke-Ringer's solution. This was the standard amount used for incubation plus another 1.5 ml. of material to be tested (whether sera or suspended cells). Since this 3 ml. was divided among 3 test rats, it was estimated that each of the animals received approximately 100 mg. of treated tumor when the fine mince was used, and (by weighing the prepared material) approximately one half to one third less when sieved cells were employed. There is no doubt that this is a rough procedure and could be refined. However, it is noteworthy that the results obtained with both types of prepared cells agreed, even though the fine mince consisted of cell clumps of relatively large size compared to the sieved tumor, which was composed of individual or small groups of cells. For the purpose of uniformity, all the charts that illustrate this paper are from experiments done with finely minced HEp 3 tumor, rather than with sieved material.

Test sera were obtained from etherized rats by bleeding them from the

heart with sterile dry needles and syringes. The blood was gently squirted into the lower base of a sterile Petri dish propped up with Plasticene. Material from several rats, similarly immunized, was pooled. After a clot had formed in the dish the latter was rotated so that the clear sera might collect. Hemolyzed sera was discarded. Although various concentrations were tested against the HEP 3 suspensions, in the experiments reported herein the amount of sera used was 0.75 ml. for each 1.5 ml. of standard tumor cell suspension plus an additional 0.75 ml. of BGR to make up the 3.0 ml. in each tube. There was thus approximately 0.25 ml. of whole sera per 100 mg. of tumor. After all the rats had been bled, each was sacrificed. Organs to be tested (including spleen, lymph nodes, or kidney) were removed, weighed, and minced very fine in BGR, so that 1.5 ml. contained 500 mg. of solid material, or a proportion of 500 mg. of test tissue to 300 mg. of tumor.

Suspensions, placed in small glass test tubes, were incubated in a water bath at 37° C. for 1 hour before they were implanted in X-irradiated test hosts. The latter received the usual additional conditioning of 3 mg. of cortisone, injected subcutaneously, at the time of implantation and 3 subsequent injections on alternate days following. Tumor nodules were usually palpated and charted on the eighth and twelfth days following implantation and the animals were sacrificed on the fourteenth day.

Results

When the transplantable human tumor HEP 3 is minced, suspended in BGR, and implanted in properly conditioned heterologous hosts, it will grow progressively in 96 to 100 per cent of the animals. If such a cell suspension should fail to grow after it had been treated with a test material such as the sera or cells from rats "immunized" with human tissues, it would seem permissible to say that substances toxic to the tumor cells had been present in, or elaborated by, the test material. If sera or similar cells from normal animals did not produce the same results, it could be presumed that such cytotoxins were peculiar only to the immunized hosts, and that a test method was thereby made available for the detection of humoral or cellular cytotoxins in such animals. To be certain that the entire testing system employed was valid, it would also be necessary to establish that the X-irradiated and cortisone-treated host in which the treated tumor cells were implanted was a "neutral" medium that would not itself produce cytotoxins and, further, that it would not retain, in an effective capacity, any that were introduced in the implanted suspension. FIGURE 1 illustrates the findings of a pilot experiment that encompassed the problems discussed. This experiment was repeated on two other occasions with similar results, and it indicated that the method proposed for the determination of cytotoxins was sound. The spleens of test hosts were used because of the work with homologous cellular cytotoxins already cited. As will be noted, the animals of this experiment were killed on the twelfth day after implantation, and the tumors present were charted after the overlying skin was deflected. Only the HEP 3 cells that had been incubated with spleen cells from immunized rats failed to grow. Tumor cells treated with minced spleen from normal rats or from conditioned hosts grew as well

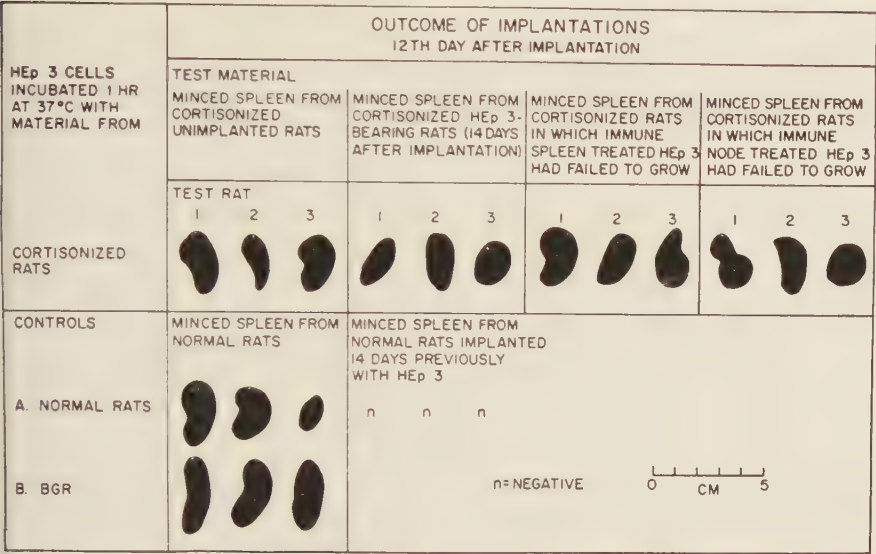


FIGURE 1. Tests with human epidermoid carcinoma No. 3 (HEp 3) cells incubated *in vitro* with spleen cells from cortisone-treated weanling rats.

as the BGR control (FIGURE 2). It is noteworthy that it made no difference whether the conditioned host was carrying tumor, had never been implanted, or had been injected 14 days previously with a mixture of the tumor and "immune" spleen or node cells; the results were the same and their spleen cells did not inhibit growth of the HEp 3 carcinoma. It would seem safe to say that the conditioned host is indeed neutral and that, under the conditions of this experiment, it does not appear to become passively immunized by the introduction of cells from immune animals when the mixture of tumor and test material is implanted.

An experiment was then devised to determine just when cytotoxins might appear in the sera or become associated with the cells of the immunized hosts. Twenty-four normal rats were divided into 6 groups of 4 each and injected 13, 10, 7, 3, and 1 days prior to the experiment with HEp 3 cells. One group of the normal rats served as controls. When the sera and lymph node plus spleen cells from the 6 groups were incubated with HEp 3 and the mixtures implanted as usual, it was learned that both humoral and cellular cytotoxins had appeared about the seventh day after immunization (FIGURE 3). The humoral cytotoxins then decreased, while the cellular cytotoxins were still effective in material from animals immunized 13 days previously. In this experiment the immunized rats were rather small weanlings, and the amount of HEp 3 mince injected into them was approximately one half the usual dose given, so that the immunity obtained was probably smaller than usual (compare the strong cellular cytotoxins present in other immunized hosts on the fourteenth day in FIGURE 1). This comparatively feeble immune reaction emphasizes the peak strength of the humoral cytotoxins at 7 days. It is interesting to note that the results were the same whether the lymph node and



FIGURE 2. Relative growth of HEp 3 incubated with spleens from either normal or immune rats prior to implantation in the conditioned rat. The 2 animals at the right were implanted in the flank with a mince of HEp 3 that had been incubated at 37°C . for 1 hr. with spleen cells from normal rats prior to implantation; the 2 animals at the left received similar amounts of a mince of HEp 3 that had been incubated with spleen cells from rats that had been immunized with HEp 3 14 days previously. The tumor grew well in the first 2 animals. No proliferation occurred in the others; the nodules seen were composed of dead debris.

spleen cells used for testing were suspended in BGR or in their own sera. This indicated that they did not have to be in their more natural medium in order to "produce" the cytotoxic effect on HEp 3 cells. Likewise, the combination of sera and cells did not appear to produce a cumulative effect.

FIGURE 4 gives the results of an experiment in which sera and cells from immunized young adult and weanling rats were compared for their cytotoxic potency against the HEp 3 cells. The 2 groups of animals had been given the usual full amount of the same immunizing material, in this instance 22, 14, 10, 7, 3, and 1 days before the experiment. Four rats of each age were injected on these days. As can be noted, material from the older rats seemed slightly more effective than that from the weanlings, but not as much as might have been expected in view of the fact that such young animals are considered to be poor producers of antibody. Again, both humoral and cellular cytotoxins appeared on about the seventh day after immunization, and cytotoxins of the sera were present for a limited period (the seventh, tenth, and fourteenth days in the young adult rats). By the twenty-second day after immunization they

HEp 3 CELLS INCUBATED 1 HR. AT 37°C WITH	OUTCOME OF IMPLANTATIONS											
	DONOR MATERIAL NOT IMPLANTED PREVIOUSLY			DONOR MATERIAL FROM RATS IMP 1 DAY PREVIOUSLY			DONOR MATERIAL FROM RATS IMP 3 DAYS PREVIOUSLY			DONOR MATERIAL FROM RATS IMP 7 DAYS PREVIOUSLY		
	TEST RAY			TEST RAY			TEST RAY			TEST RAY		
	8 12			8 12			8 12			8 12		
I. SERA	1		↑	1		12		12		n	n	n
	2		↑	2		12		12		n	n	n
	3		↑	3		12		12		n	n	n
II. MINCED LYMPH NODES AND SPLEEN PLUS SERA	1		↑	1		12		12		n	n	n
	2		↑	2		12		12		n	n	n
	3		↑	3		12		12		n	n	n
III. MINCED LYMPH NODES AND SPLEEN + SERA	1	↑	↑	1		12		12		n	n	n
	2		↑	2		12		12		n	n	n
	3		↑	3		12		12		n	n	n
IV. BOR CONTROL	1	↑	↑	1		12		12		n	n	n
	2		↑	2		12		12		n	n	n
	3		↑	3		12		12		n	n	n

FIGURE 3. Tests with HEp 3 cells incubated *in vitro* with sera and/or lymph node and spleen cells from weanling rats immunized with HEp 3.

HEP 3 CELLS INCUBATED 1HR AT 37°C WITH MATERIAL FROM I. YOUNG ADULT RATS	OUTCOME OF IMPLANTATIONS 14TH DAY AFTER IMPLANTATION																							
	DONOR MATERIAL FROM NORMAL RATS			DONOR MATERIAL FROM RATS IMP 1 DAY PREVIOUSLY			DONOR MATERIAL FROM RATS IMP 3 DAYS PREVIOUSLY			DONOR MATERIAL FROM RATS IMP 7 DAYS PREVIOUSLY			DONOR MATERIAL FROM RATS IMP 10 DAYS PREVIOUSLY			DONOR MATERIAL FROM RATS IMP 14 DAYS PREVIOUSLY			DONOR MATERIAL FROM RATS IMP 22 DAYS PREVIOUSLY					
	TEST RAY			TEST RAY			TEST RAY			TEST RAY			TEST RAY			TEST RAY			TEST RAY					
	1 2 3			1 2 3			1 2 3			1 2 3			1 2 3			1 2 3			1 2 3					
A. SERA	1		2	1		2	1		2	1		2	1		2	1		2	1		2	1		2
B. MINCED NODES AND SPLEEN	1		2	1		2	1		2	1		2	1		2	1		2	1		2	1		2
C. MINCED NODES AND SPLEEN PLUS SERA	1		2	1		2	1		2	1		2	1		2	1		2	1		2	1		2
II. WEANLING RATS	1		2	1		2	1		2	1		2	1		2	1		2	1		2	1		2
A. SERA	1		2	1		2	1		2	1		2	1		2	1		2	1		2	1		2
B. MINCED NODES AND SPLEEN	1		2	1		2	1		2	1		2	1		2	1		2	1		2	1		2
C. MINCED NODES AND SPLEEN PLUS SERA	1		2	1		2	1		2	1		2	1		2	1		2	1		2	1		2
BOR CONTROL	1		2	1		2	1		2	1		2	1		2	1		2	1		2	1		2

FIGURE 4. Tests with HEp 3 cells incubated *in vitro* with sera and/or lymph node and spleen cells from rats immunized with HEp 3.

could no longer be detected in these hosts, although some still remained in the younger animals. In contrast, the minced nodes and spleens of the adult rats were highly cytotoxic for the HEp 3 cells even in rats immunized 22 days prior to the experiment.

A test was then made of the cytotoxic properties of sera and cells from "hyperimmune" rats as contrasted with material from animals given only the usual single injection of HEp 3. Twelve normal young adult rats were each given 2 injections of the tumor 10 days apart. Fifteen days after the second injection these animals were divided into 3 groups of 4 rats each and, together with 3 similar sets of normal rats not previously injected, were implanted with HEp 3 cells 10, 7, and 3 days prior to the experiment. One group of 4 hyperimmune and 2 groups of normal rats were not injected at this time. Sera, as well as lymph node, spleen, and kidney cells from both types of immunized animals were tested against the HEp 3 cells. Results of this experiment are given in FIGURE 5. As will be seen, the cytotoxic effects of sera from rats immunized once were apparent only in the animals immunized 10 days previously. On the other hand, cytotoxins were detected 7 days after immunization in the hyperimmune hosts. The fact that in the experiments of FIGURES 3 and 4 humoral cytotoxins were present on the seventh day after a single injection of HEp 3 cells, and not until the tenth day in the experiment of FIGURE 5, might be due to some variation in the particular immunizing inoculum used. The important finding is that hyperimmune animals respond more quickly with humoral cytotoxins than rats injected only once. Minced lymph node and spleen cells from both groups of animals were effective as early as 3 days after injection of the HEp 3, although the cytotoxins associated with the lymphoid cells of hyperimmune rats seemed the more potent. Of special interest was the finding that minced kidney cells from any of the animals were without deleterious effect. Another experiment, not charted here, confirmed this latter observation. Minced muscle, heart, liver, kidney, or lung from HEp 3 immunized rats did not contain or produce cytotoxins for these tumor cells, although spleen, lymph nodes, and also thymus were quite effective in inhibiting their growth after they were incubated together. A possible exception might be lung, which showed some questionable results (could this be due to the large number of lymphoid cells present in this organ?).

To determine whether the cytotoxins produced were entirely antihuman or somewhat tumor-specific, rats were immunized with 3 different transplantable human neoplasms. Four rats received single injections of tumor 7 days before the experiment, and 4 others each received 2 (one 16 days, one 7 days prior to the test). The latter animals were considered hyperimmune. Material from them was labeled "16-day hyperimmune" in FIGURE 6. It will be noted that animals injected with HEp 3 cells produced the strongest cytotoxins to this tumor. Both the sera and lymphoid cells were highly effective, whether their donors had received 1 or 2 immunizing injections. Material from rats immunized with HEp 4 was not potent when obtained from animals given 1 injection, but it was quite effective when it was taken from those that had received 2 injections. The lymphoid cells, in particular, were highly cytotoxic.

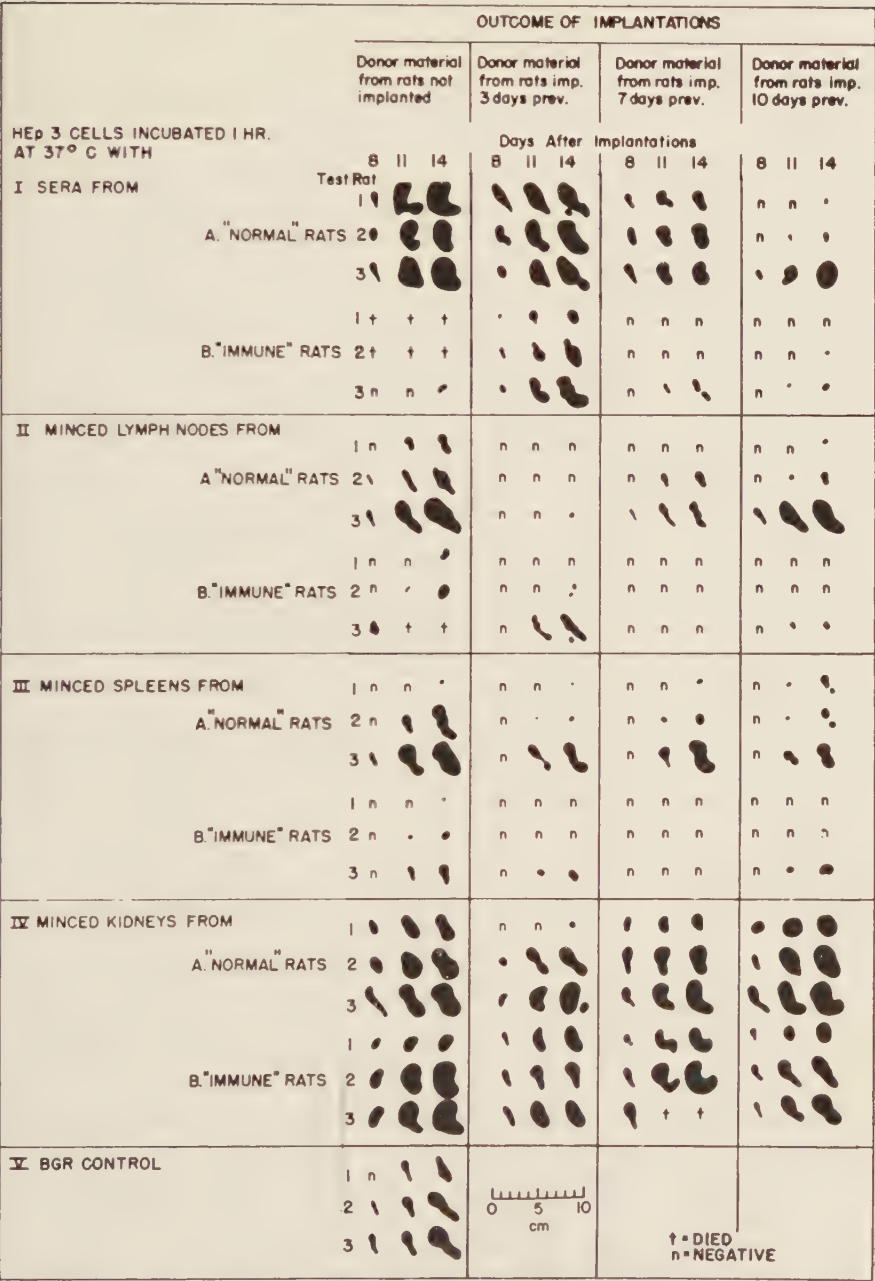


FIGURE 5. Tests with HEP 3 cells incubated *in vitro* with sera or cells from rats immunized with HEP 3.

HEP 3 CELLS INCUBATED 1HR. AT 37° C WITH MATERIAL FROM RATS IMMUNIZED WITH A CELL SUSPENSION OF:		OUTCOME OF IMPLANTATIONS 14TH DAY AFTER IMPLANTATION																	
		TEST MATERIAL			7 DAY MINCED NODES AND SPLEEN			7 DAY MINCED NODES AND SPLEEN + 7 DAY SERUM			16 DAY HYPERIMMUNE SERUM			16 DAY HYPERIMMUNE MINCED NODES AND SPLEEN			16 DAY HYPERIMMUNE MINCED NODES AND SPLEEN + 16 DAY SERUM		
		TEST RAT																	
1. Hep 3		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2. HS 1																			
3 Hep 4																			
CONTROLS:	SERUM	MINCED NODES AND SPLEEN			MINCED NODES AND SPLEEN PLUS NORMAL SERUM														
A. NORMAL RATS																			
B. BGR																			

n = NEGATIVE
+ = DIED

0 CM. 5

FIGURE 6. Tests with HEP 3 cells incubated *in vitro* with sera and/or lymph node and spleen cells from rats immunized by 3 different transplantable human tumors.






















HEp 3 CELLS INCUBATED 1 HR. AT 37° WITH MATERIAL FROM RATS IMMUNIZED WITH:	OUTCOME OF IMPLANTATIONS 14th DAY AFTER IMPLANTATION								
	TEST MATERIAL			11 DAY MINCED NODES			11 DAY MINCED THYMUS		
	TEST RAT								
1. WHOLE HEp 3 CELLS	1 	2 	3 n	1 	2 n	3 n	1 n	2 n	3 n
2. DISINTEGRATED HEp 3 CELLS		n	n	n	n	n		n	n
3. WHOLE HS 1 CELLS			n		n	n	n	n	+
4. DISINTEGRATED HS 1 CELLS						n			
CONTROLS	SERUM			MINCED NODES			MINCED THYMUS		
A. NORMAL RATS									
B. BGR				n = NEGATIVE + = DIED			0 CM. 5 		

FIGURE 7. Tests with HEP 3 cells incubated *in vitro* with sera or cells from rats immunized with whole or disintegrated human tumors.

Apparently, immunization with HS 1 produced very weak cytotoxins, either humoral or cellular. The results of this experiment are not unexpected. Korngold^{19, 20} has shown that, in human tissues, there are present many antigens that are not shared by all hosts. The transplantable human tumors that he has tested by the Ouchterlony plate method, discussed elsewhere in these pages by Pierre Grabar, differ among themselves in the number and type of the antigens that they contain. Therefore it is not surprising that some human tumors may prove better "immunizers" than others. It is still noteworthy, however, that HEp 3-immunized hosts produced strong cytotoxins to these cells, while animals immunized with a tumor of similar type and origin (HEp 4) were much less effective in doing so.

Since Billingham, Brent, and Medawar²¹ have noted that disintegrated cells will retain their power to elicit skin transplantation immunity in mice, an attempt was made to determine whether HEp 3 cells that had been disintegrated by ultrasonic irradiation also would be able to stimulate the production of cytotoxins in rat hosts. FIGURE 7 shows that they were. Eleven days after rats had been immunized with either whole or disintegrated HEp 3 cells their sera and lymphoid cells (nodes and thymi, in this case) were toxic to HEp 3. Material from animals that had been immunized with twice the usual dose of HS 1 cells was also tested. Again, it was not as effective as that from the HEp 3-immunized hosts, but undoubtedly some cytotoxins had been produced. It appears that in this instance the disintegrated tumor had not stimulated the rat hosts as much as the whole cells. It is interesting to observe that cells from the thymus were just as effective as those from the lymph nodes and spleen in inhibiting the test tumor. This finding may be related to Stoner's observation²² that thymi are able to produce antitoxins.

In brief, the experiments reported indicate that both humoral and cellular cytotoxins can be detected by the means outlined. Both types of cytotoxins become apparent in the heterologous host approximately 7 days after an immunizing injection and, under the conditions of this experiment, they are of greater potency and longer duration in the cells than in the sera. No attempt has been made to define the nature of these substances. It may be that they are the same in both sera and cells. The present paper has been concerned only with the detection of their presence. It will be interesting to discover in experiments now in progress (1) whether there is an ideal "antigen-antibody" (HEp 3 cells-cytotoxin) ratio for incubation that will give more definitive results than those noted, (2) whether there are cross reactions between various other transplantable human and animal tumors as well as normal human tissues, and (3) in what fraction of the cells the immunizing power lies. Will it be in the deoxyribonucleoproteids, as Billingham believes?²¹ Work on the problem of cross reactions is probably the most difficult, as normal human tissues are still cultivated in limited quantity. Since uniform material must be used, fresh normal tissue could be obtained only from the same donor, in view of diverse antigenic make-up among humans. Obviously, this presents difficulties. The role that complement may play in these reactions is also being investigated.

It is possible that even homologous cytotoxins might be studied and evalu-

ated by use of the conditioned rat. Certainly, it affords a means of testing mouse sera and cells against a homologous tumor in an unrelated host medium.

Summary

Cytotoxins for the transplantable human tumor HEP 3 have been detected in the sera and associated with the lymphoid cells of rats immunized with either whole or disintegrated cells of this neoplasm. Under the conditions of the experiments reported herein, such cytotoxins were neither present in similar material from normal rats nor associated with tissue other than lymphoid (nodes, spleen, thymus) of the immunized animals. The cellular cytotoxins appeared to be more potent than those in the sera, and they were detected in the immunized hosts for a longer period of time.

X-irradiated and cortisone-treated rats proved a usable "neutral" test medium for the determination of damage to minced or sieved tumor that had been treated and incubated with test sera and/or cells.

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DISCUSSION: PART IV

Peyton Rous, *Chairman*

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EUGENE D. DAY (*Roswell Park Memorial Institute, Buffalo, N. Y.*): Robert Wissler has informed me that he will soon be testing the localization of anti-Ehrlich antibodies in the solid-ascites form of the Ehrlich tumor as a means of paralleling the type of work that we have done in our laboratory. I feel quite sure that, in this particular case, he may achieve localization as high as or higher than we have in the solid-ascites form of the Murphy rat lymphosarcoma.

I should like to ask Robert Wissler this question: What evidence is there that capillaries in the vascular beds of certain tumors are unable to pass gamma globulin molecules from the circulation into the extracellular fluid of the tumor? If such passage is not possible, it could be a source of considerable difficulties in any future work on tumor localization.

ROBERT W. WISSLER (*Department of Pathology, University of Chicago, Chicago, Ill.*): I thank Eugene Day for his comments. I am afraid I cannot add very much to what he said except to state that our impression is that gamma globulin must have some difficulty penetrating the vascular walls since we are able to show definite *in vitro* tumor localization of antitumor gamma globulin using lyophilized tumor cells, and we cannot demonstrate this *in vivo* when we inject the labeled tumor antibody intravenously. Our assumption is that the capillary membrane is blocking the passage of this material into the tumor cells. Our experience with the ascites tumor supports this assumption.

I hope, as you do, that antibody molecules to tumor may be localized in the tumor cell of the solid tumor *in vivo*. I think it is evident from our work that if they do get to the tumor cell in sufficient quantity, as they do in the case of the ascites tumor, then they can produce remarkable effects both on the growth and metabolism of the tumor cell.

W. J. NUNGESTER (*Department of Bacteriology, University of Michigan, Ann Arbor, Mich.*): I am greatly impressed by the fine contributions to this monograph by John Ross and by Robert Wissler and Martin Flax. Both of these papers led up to the problem of the permeability of the capillary by the immune globulins. In our own laboratory, during the last several years, we have obtained an increasing body of evidence showing that the larger protein molecules do not pass through the capillary walls in tumor-bearing tissue. However, we have recently found pharmacological means of increasing the permeability of the capillary by the use of such drugs as niacin and serotonin. I should like to ask if these investigators have had any experience with the pharmacological approach to capillary permeability.

JOHN D. ROSS (*Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minn.*): No, I have not. I have been concerned largely with a method of assay of cytotoxic antibody. Certainly, if anything of this nature offers any hope of finding a solution to the problem of permeability, it merits investigation.

JOSEPH W. BEARD (*Duke University School of Medicine, Durham, N. C.*): It is not my intention to question the results reported by Harry Rubin in the section *Results of Immunological Techniques*, nor to indulge in controversy over his interpretations relative to the properties of the Rous sarcoma virus. Nevertheless, there are profound objections to the application of observations on the chicken sarcoma to those concerned with the antigenic constitution of the avian leukemias, erythroblastosis, and myeloblastosis. Further experiments with these two leukemia viruses have uncovered no evidence of reversibility of the union of virus with antinormal chick-tissue rabbit immune bodies established on incubation *in vitro* either by additional dilution *in vitro* or by the dilution that occurs when the virus-serum mixture is injected into the host. In addition, immune serum inoculated separately from the virus has no neutralizing effect on the agent, whether it is injected immediately before or immediately after the administration of the virus. In these respects the heterologous rabbit immune serum did not behave differently from the specific homologous antiviral immune bodies from the chicken. It is quite apparent that the behavior of the leukemia viruses is entirely different from that of the Rous sarcoma virus, and that the results obtained with this agent cannot be used to discount the evidence that an antigen that behaves as does normal chick-tissue protein is an integral constituent of the leukemia viruses.

KARL HABEL (*National Institute of Allergy and Infectious Diseases, Public Health Service, Bethesda, Md.*): In some instances the low titers of cytotoxic activity of rabbit sera may be explained on the basis of a normal toxic component of such sera, as has been suggested. However, I do not believe that the rabbit sera currently used in our studies could have contained any of these normal toxic substances, since our tissue cultures were grown in a medium that contained 20 per cent normal rabbit sera. Excellent cell growth was obtained in this medium. Furthermore, the fact that the cytotoxic titers of our antisera were reduced after adsorption with red blood cells is evidence that indicates that the cytotoxic effect was due to adsorbable antibody.

ALEX B. NOVIKOFF (*Waldemar Medical Research Foundation, Port Washington, N. Y.*): I should like to ask Brent how he would place the antigens involved in resistance to tumor transplantation in his second group. From the earlier work of MacDowell,² Kidd,³ and Snell⁴ on the development of resistance by means of cell-free extracts, from Barrett's⁵ work with red cell stromata, and from observations of our own⁶ with microsome fractions completely free of nuclei and of deoxyribonucleic acid (DNA), it is difficult to conceive that antigens involved in the production of resistance are restricted to nuclei or to DNA in such cases.

MORRIS K. BARRETT (*National Cancer Institute, Public Health Service, Bethesda, Md.*): As many of you would expect, I have more to say at this point than I have space to express it. However, I shall try to make a point or two. Novikoff has already touched upon something that is very closely related to what I wish to emphasize. We have assumed for many years that the type of immunity represented by reaction to normal tissues and that type of immunity which is represented by resistance to implantation of a tumor were the same, at least generally. There is no evidence from a half century of investigation,

to my knowledge, that indicates real difference between these two types of immunity, unless the present discussion points to such a difference.

A striking characteristic of immunology is its controversial nature. There are so many paradoxical things in this area of investigation! If we seize on one particular paradox it may lead to something worth while. I have been familiar with the work of Brent, Billingham, and Medawar, as I think they have been with mine, and I have never seen anything that is as completely irreconcilable as the difference between our results and theirs, provided one assumes that there is no fundamental difference between resistance to tumor and resistance to skin; perhaps that is the point. If so, it should be pursued.

Novikoff has just pointed out to you that I can get a high grade of resistance in mice against the implantation of a tumor with merely the washed stromata of a red cell. I do not know how one would go about finding nucleic acid or nucleoprotein in such material. Furthermore, when we turn to the special qualities of my tumor or mice, we remain baffled. The general phenomenon I have reported does not apply to only the one system that I have been reporting lately. It applies to 6 or 8 strains of mice, and to 6 or 8 different kinds of tumors, and it has been observed in more than one laboratory. I do not understand how two investigators can be as far apart as Brent and I are unless the difference is significant of something.

As I said about a year ago,⁷ there must be some key concept that we have missed and, if we keep emphasizing such differences, we may finally come upon a new key concept.

To make matters worse, I get contrary results, not only with the red cells, but also with cell products. For instance, if I take tumor as my immunizing agent and subject it to the same 9-kc. high-pitched audible sound (that is, not supersonic sound) that I use for red cells, and if I inject the entire product as antigen, I obtain no resultant immunity. Presumably, such a preparation would contain any nucleic acid, nucleoprotein, or other reasonably stable cell product that was in these cells, and it should be in a relatively natural state and dosage.

VAN R. POTTER (*University of Wisconsin School of Medicine, Madison, Wis.*): I address my question to Leslie Brent. It is my understanding that preparations from any organ will immunize against skin in your transplantation system. Am I correct in this assumption? You have said that you can take any organ from animal A, make a preparation from it, inject this preparation into animal B, and thus cut down the survival time of a skin graft from animal A to animal B. Did I understand you correctly?

LESLIE BRENT: No, we have not applied our findings so generally. Our work on nonviable antigenic preparations has been confined entirely to the spleen and the thymus, for purely practical reasons.

VAN R. POTTER: It was my impression that in your article in *Nature*⁸ you generalized your observations.

LESLIE BRENT: No. We stated our findings very clearly.

VAN R. POTTER: However, you generalized the concept, the idea. Is it your opinion that, from a theoretical standpoint, any tissue will produce this result?

LESLIE BRENT: Yes, this seems to be indicated theoretically.

VAN R. POTTER: If this is true, and if you draw certain conclusions from the fact that each tissue has the same complement of DNA, then will killed tumor cells or a DNA preparation of such killed cells immunize animals against skin transplants?

LESLIE BRENT: Do you refer to tumor preparations that have been disintegrated in the same manner as our spleen or kidney cells?

VAN R. POTTER: Yes, I think that this is true.

LESLIE BRENT: I cannot give you an unequivocal answer, since we have not made any experiments of this nature. However, I do not believe that this would be true. It would be most interesting to know the answer.

VAN R. POTTER: Might you predict what would happen? What would you conclude from either positive or negative results from such experiments?

LESLIE BRENT: I would predict that immunization would be effective.

ROBERT A. GOOD (*Department of Pediatrics, The Medical School, University of Minnesota, Minneapolis, Minn.*): Perhaps I overlooked an important point in Brent's presentation. Certainly he has demonstrated clearly that there is a transplantation antigen that is linked to the nuclear material or that is part of it. This has been established clearly by his excellent data. On the other hand, I am not convinced that he has shown that all of the antigens that are involved in the transplantation reaction are located in the nuclear materials. It would be interesting to know whether he has attempted to produce acquired tolerance with the antigen that he has described. If it were possible to produce immunity in this manner, his argument would be complete, and it would be demonstrated that antigens that have cytoplasmic locations play no important role in the failure of transplantation. However, if tolerance cannot be produced with these antigens, it would seem possible that other antigens, perhaps even some of those that are in the cytoplasm, might contribute to the rejection of homotransplants.

LESLIE BRENT: We have injected suspensions of nuclear fibers into newborn mice by the intravenous route, which we know to be effective when living spleen cells are injected. The preliminary results indicate that any tolerance that is brought about in this way, if it occurs at all, is of a very trivial nature. These experiments are still in progress, however. My colleagues Medawar and Billingham are, at the moment, injecting much larger doses of nuclear fibers, as the earlier results may have been due to a dosage factor. However, it is fair to say that up to this time it has not been possible to produce anything approaching permanent tolerance by the injection of nonviable nuclear preparations.

DANIEL G. MILLER (*Memorial Center for Cancer and Allied Diseases, New York, N. Y.*): Is it possible that Morris Barrett's cell membranes and your nuclear membranes may have some antigens in common?

LESLIE BRENT: I have no ready answer to this question. We are confronted with two sets of totally different data which, superficially at least, it is impossible to reconcile. However, it may be fair to point out that Barrett's system differs from ours insofar as he has been using tumors as test grafts. Even more important is the fact that the animals used as hosts in his experiments

belong to a strain 10 per cent of the members of which will normally display resistance to an implanted tumor even when they have not been immunized previously. This suggests that the other 90 per cent of these animals may also possess a weak, subthreshold resistance. Is it not possible that such resistance could be accentuated by nonspecific methods?

MORRIS K. BARRETT: All of this is true, but that is one of the points that I wished to emphasize. While the system that I reported in recent papers was, indeed, a 90-10 per cent system, I have also worked with other tumors, and some of these "take" in only 30 per cent of the mice, and others do so in all of them. Although all of the experiments were not alike, the phenomena do not appear to be limited to one particular tumor.

SIDNEY SCHULMAN (*Department of Bacteriology and Immunology, University of Buffalo, Buffalo, N. Y.*): I should like to ask Brent whether it is not surprising that the freezing treatment should inactivate this material if we ascribe its activity to nucleoprotein. I wonder if the possible action of lipid should not be considered, since lipoproteins are known to be damaged by freezing, probably more generally so than are nucleoproteins. One might investigate this possibility by attempting to remove the lipid by means of cold organic solvents or, possibly, by further extensive purification of the nucleoprotein material; for example, this could be done by repeated precipitation at high and low salt levels. It also might be of interest to add that the erythrocyte membrane would be expected to contain lipid.

LESLIE BRENT: We have considered this possibility, and we have tentatively suggested, in our paper in *Nature*, that the antigens may be lipoproteins. The great instability of the antigenic preparations would certainly fit such an hypothesis.

HELENE W. TOOLAN (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): I should like to raise the question as to whether transplantable human tumors remain human when grown in conditioned animals. My colleague Leonhard Korngold has been using the Ouchterlony plate technique to test our human transplantable tumors for their antigen type. He has examined these tumors from the time of their first and second generations on through their one-hundred-and-twentieth generation, and he has found that their antigen type has remained constant and remains human.

I should also like to make another point; namely, that we have found no correlation between the degree of malignancy of a given tumor in the patient and its ability to grow in conditioned hosts.

FREDERICK C. CHESTERMAN (*Bland-Sutton Institute for Pathology, Middlesex Hospital, London, England*): I should like to ask Harry Greene if his relation of homotransplantability to metastasizability always applies to breast cancers. I ask this because I have had great difficulty in growing breast cancers in brain tissues, but none at all in growing the oat cell carcinoma in the same medium.

HARRY S. N. GREENE (*Yale University School of Medicine, New Haven, Conn.*): If one defines metastasis as discontinuous growth, the relationship between metastasizability and transplantability holds in all tumors. Your failure to transplant breast cancer means that, at the time of transfer, the tumor had not yet attained the ability to metastasize. Let me emphasize

that, in the great majority of cases, the involved axillary nodes in breast cancer represent lymphatic extensions and not metastases. On the other hand, your successful transfer of oat cell carcinomas of the lung means that the tumor in question had attained metastasizability at the time of transfer. Confirmation of this point will be obtained if you investigate the fate of the patients concerned in your transplantation experiments.

In answer to the comments of Helene Toolan, it should be emphasized that our differing results must be interpreted on the basis of the different techniques employed. Toolan uses recipient animals whose reactions have been modified by cortisone or X irradiation prior to transfer, while my animals are normal and untreated. She has shown that human tumors will grow in her treated animals, irrespective of the developmental stage of the tumor at the time of transfer. On the contrary, only tumors in the final developmental stage of metastasizability will grow in my normal animals. This raises a point relevant to much of this discussion. It would appear that among biochemists and immunologists, at any rate, there exists an assumption that tumors are static entities endowed from inception with all of the biological attributes that characterize malignancy. This is emphatically not the case. The qualities that distinguish cancer are not present from inception, but represent developmental acquisitions. In other words, cancer is not a sudden transformation of normal cells but, on the contrary, it represents the final phase in a developmental process. Accordingly, investigations concerning the biochemistry or immunology of a tumor require characterization of the tumor from a biological point of view before a rational interpretation of the results becomes possible.

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SUMMARY

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Perhaps the major contribution of this monograph will be to focus attention upon the important influences of host-tumor relationships at a time when extensive efforts are in progress to find chemicals that will destroy cancer cells in a manner similar to the destruction of bacteria by antibiotics. The quality of the papers presented and the interest of the contributors are ample evidence that a goodly number of investigators expect that a better understanding of the cancer process, especially the reactions between it and its host, will contribute to control of the disease.

The chief purpose of the conference on which this monograph is based was to explore the reactions of the host to the development and growth of malignant cells, and there are reasons for believing that the host does exert some influence in both respects. Within highly inbred strains of experimental animals there is a remarkable variation in the response of individuals to a uniform carcinogenic stimulus. Differences between the rates of growth of established tumors and their tendencies to metastasize, together with the rare occurrence of spontaneous regression, justify continued investigations of the host's resistance to the cancer process.

Probably the best procedure in attempting this summary is to formulate briefly the current status of the problem and to review the progress reported in these pages. I can think of no better place to begin than with the classic review by Woglom¹ published in 1929. This review dealt chiefly with immunity to transplantable tumors, but Woglom's presentation of the problem was such that it serves our purpose admirably. It summarized progress during the first twenty-five years of this aspect of cancer research: immunity to cancer has been studied for about fifty years. It can therefore be used as a framework for this summary and to reveal the progress achieved during the intervening years.

Woglom discussed the problem under the two general headings of natural and acquired immunity. This approach was for the sake of convenience, because he considered the two types of immunity as differing only in degree.

Early Concepts of Natural Immunity

Natural immunity was divided into 3 types: immunity of foreign species, racial immunity, and individual immunity. The first, which we now refer to as immunity to heterologous tumors, had received considerable attention, and it was the consensus that adults of a foreign species were completely immune to the "continued proliferation" of a subcutaneous graft. However, it was known that such grafts could survive for 54 days and that a variety of procedures abrogated, partially at least, the resistance of heterologous hosts. Parabiosis, splenectomy, hormones, and blockade of the reticulo-endothelial system were effective. The site of inoculation was known to be of importance, because the eye, brain, and developing chick embryo supported heterologous

growths better than other sites. The age of the host was explored; while animals less than 24 hours old were known to be most susceptible, tumors growing in them did not adapt to adults. The lymphocyte was deeply implicated as the cell chiefly responsible for the hosts' natural resistance.

Racial immunity included the difference in susceptibility between members of the same species, and here Woglom reviewed the findings of investigators who used animals from different sources or who worked in different localities with the same transplantable tumor. These differences were regarded as reflecting differences between the genetic constitutions of the hosts.

Individual immunity included those factors that determined the susceptibility of the individual animal to a particular tumor. The age of the host and local factors at the inoculation site, such as irritants and hyperemia, influenced the success of the inoculum. Pregnancy and hormonal stimulation were effective. Of most interest to us are the facts that pioneer work on the influence of heredity in determining susceptibility of the host to tumor growth had been accomplished and that the genetic rules for transplantation of tissues had been formulated, although they were not widely known.

Under the general heading of acquired immunity Woglom included the resistance to inoculation following the regression of a grafted tumor or after the tumor had been removed surgically. Natural immunity also is elicited in animals that have been treated with normal tissues or organs. Most investigators considered embryo skin to be the most effective of these, but a variety of organs and tissues were able to evoke resistance to the growth of transplantable tumors.

Early Concepts of Acquired Immunity

Problems of acquired immunity had been studied diligently for a number of years before Woglom's review because of the successful application of serological and immunological techniques to the control of a number of other diseases. Time does not permit the attention these efforts should receive, so I shall state briefly ten phases of this work upon which there was general agreement and which have a direct bearing upon the subjects included in this monograph.

(1) Acquired immunity was dependent upon the immunizing material injected and the tumor used in the test for immunity. There was considerable variation in the immunizing abilities of tissues and in the abilities of different tumors to overcome such acquired resistance. Regression of a transplantable tumor usually, but not always, rendered the animal immune to further growth of the same tumor, but some tumors were far more effective than others in this respect. Immunity following regression of a tumor could be specific for the tumor or extend to other tumors. These complexities of host-tumor relationships were as baffling to our predecessors as any discussed in this publication.

(2) Living intact cells were thought to be essential for the production of immunity.

(3) Concomitant immunity was recognized. This type of immunity is induced by a tumor while growing in its host, but it exerts no demonstrable influence upon the growth of the immunizing tumor. This facet of the problem had been explored thoroughly and, in common with many problems in cancer

research, had proved to be very complex. The temptation to search for a tumor capable of producing immunity to all types of tumors must have been very strong.

(4) It was considered impossible to immunize animals against their own spontaneous tumors.

(5) Propagable tumors and transplanted normal tissues obeyed the same general rules of transplantation.

(6) The virus-induced Rous sarcoma was studied and was found to evoke two separate kinds of immunity: one against the tumor cell and the other against the virus.

(7) A specific antibody against any tumor had not been found.

(8) A specific antigen within any tumor had not been found.

(9) Cytotoxins effective against tumor cells were known.

(10) Growth-enhancing factors capable of inducing "hypersusceptibility" to growth of tumors had been found in tumor extracts.

Woglom closed his review with the following sentence: "Nothing may accordingly be hoped for at present in respect to a successful therapy from this direction." A similar pessimistic attitude is found in some later reviews of the same subject. However, it is clear that this attitude has not been too harmful. First, the attendance at the conference on which this monograph is based indicated a lack of discouragement. Second, a discouraging outlook is always a challenge to tough-minded scientists, especially to those of the next generation. Third, it arouses a strong desire to know why all previous efforts failed. However, of more importance is the record of progress since Woglom's review, which I shall now attempt to summarize.

Natural Immunity Today

Much progress had been made in overcoming the natural resistance of foreign species. Abrogation of this resistance has been extended by X irradiation and by cortisone or by a combination of both. We have learned how the techniques of eye and brain inoculation have been extended and shown to be most suitable for growth of spontaneous tumors that have acquired a high degree of malignancy.

Developments in the production of acquired tolerance fit into this part of our summary. The fact that the administration of a tissue to animals during fetal growth induces a state of tolerance to the same tissue in adult life is a development of recent years and holds promise of significant contributions to knowledge of the immunology of tumors. In these pages we read that tolerance to skin homografts can be induced by intravenous injection of spleen cells into newborn mice up to twenty-four hours of age. Of special importance was the finding that mice of certain genetic constitutions may react unfavorably to the treatment in later life. Perhaps the most important progress reported is the adaptation of mouse and rat tumors to heterologous hosts. If this evidence of a permanent adaptation is confirmed, it marks a decided advance over the consensus at the time of Woglom's review, for adult animals are no longer immune to the "continued proliferation" of heterologous tumors.

Remarkable progress has been achieved in the field of racial immunity.

Geneticists have developed strains of inbred animals that have contributed much to cancer research. For the purpose of this monograph one can think of three outstanding contributions:

First, inbred strains of animals exhibit a remarkable uniformity in their response to the growth of propagable tumors. This reduces one of the most exasperating variables with which the earlier workers had to contend. Almost every paper presented here describing the use of mice as test animals deals with inbred animals. There is little necessity to belabor this obvious advance.

Second, the availability of inbred animals has enabled us to define immunological problems more clearly. With all animals of one inbred strain that are susceptible and all animals of another strain that are immune to the growth of a particular tumor, the investigator is in an excellent position to explore immunological factors.

Third, and probably most important, is the availability of inbred strains susceptible or resistant to the development of spontaneous tumors. Some consider the use of spontaneous tumors as the crux of the immunological approach to the cancer problem, and these workers certainly have their opportunity now. Evidence has accumulated in recent years that sarcomas induced by methylcholanthrene possess the ability to elicit resistance in their strains of origin.²⁻³ This finding could be the first opening in the problem of immunity to spontaneous tumors, for it will be recalled that at the time of Woglom's review it was considered impossible to immunize an animal against its own spontaneous tumor. This goal has not been attained, but the work with methylcholanthrene-induced sarcomas suggests treatment of the tumor instead of the host in order to explore further this problem of host-tumor relationship.

Geneticists have done far more than develop inbred strains of mice for studies in tumor immunity. They have discovered that certain genes determine susceptibility and resistance to tumor transplants. We have read of further progress in this respect in that appropriate breeding of inbred mice results in the acquisition of strains possessing a low degree of resistance to transplantable tissues. This enables the investigator to conduct quantitative studies of techniques used for inducing immunity.

Another paper reveals the usefulness of inbred animals in cytologic studies. There is a correlation between the chromosomal make-up of tumor cells and their ability to grow in homologous hosts. A diploid tumor usually is strain specific, whereas polyploid tumors usually grow in a wider range of hosts. This difference suggests the possibility of revealing antigens responsible for the different degrees of specificity.

Additional knowledge has been accumulated concerning the natural immunity of the individual. Six contributions, in addition to those described by Woglom, are mentioned briefly:

(1) Dietary factors, especially those influencing the development of hepatomas.

(2) Knowledge of growth factors which, according to Woglom, induce a state of hypersusceptibility, has been extended. Filtrates of certain tumors enhance the susceptibility of the host. Substances in tumor extracts that increase host susceptibility have been defined more precisely. Lyophilized

tissues, normal and malignant, increase or decrease susceptibility depending upon dosage and other factors.⁴

(3) Cortisone and X irradiation influence the susceptibility to tumor grafts.

(4) Hormones have received much attention. Some tumors are dependent upon hormonal stimulation for growth in new hosts. Grafts of such tumors will lie quiescent within the subcutaneous tissues of an animal for as long as six months and will begin to grow only when the host receives hormonal stimulation. Tumors of this kind are called dependent tumors⁵ and have opened a new field to the immunologist interested in cancer.

(5) Growth properties of transplantable tumors can be altered by residence within the tissues of a new host. Thus certain tumors, when exposed to the influence of a new host, exhibit a pronounced change in their capacity to grow progressively in other hosts.⁶ This could be a fruitful field of exploration by interested immunologists.

(6) Transplantable tumors have been used to study the entire range of influences in both tumor and host that are involved in the process of malignancy. These factors have also been studied in induced and spontaneous tumors, and the term "tumor progression" is now used to designate the acquisition of new properties by tissues and cells as they undergo changes occurring during the malignant process.⁷ In the broadest sense such studies represent the factors influencing the entire problem of host-tumor relationship.

The last three advances could not be reviewed in detail in this work because the primary purpose of this publication has been to evaluate immunological techniques when applied to cancer research, but still the problems involved remain a challenge to the immunologist. Perhaps the complexity of tumor antigens and antibodies accompanies, or is the result of, changes appearing within cells as they undergo the change to malignancy. Hence, a comparison of immunological findings made during the early and final stages of the process may clarify some of the complexities.

Another possibility is that the factors influencing resistance to the development and growth of spontaneous tumors are not those usually found in other diseases, especially those initiated by bacteria. Hence the application of immunological techniques to the cancer problem may include new discoveries or the development of new concepts. This would not imply a cessation of efforts along classic lines, because discoveries and concepts arise through thought and effort expended on older problems.

An encouraging aspect of most advances made since 1929 is the fact that they emphasize the responsiveness of tumor cells to their environment; every new observation along these lines brings us closer to control of the disease.⁸

Acquired Immunity Today

Advances in studies of acquired immunity cannot follow the structure of Woglom's review as closely as progress in the problems of natural immunity. His review established the over-all problems so clearly that most advances have contributed to their refinement, as well as to their solutions.

The development of inbred strains has been of great value in the study of

acquired immunity. When inbred strains of mice are inoculated with established transplantable tumors they show a wide range in their degrees of susceptibility.⁹ The elucidation of the immunological factors responsible for such variable responses has not been defined. Inbred strains have also been used to establish more firmly the similarities between transplantation of normal and malignant tissues. The use of inbred animals in immunological studies has resulted in the introduction to science of the new term "immunogenetics."

Several advances in the immunology of cancer are reported in these pages. One of them consists of evidence that living intact cells are not essential to elicit immunity. The stroma of blood cells are capable of inducing resistance to a transplanted tumor,¹⁰ and a paper presented here describes the production of immunity against skin grafts by the injection of disintegrated spleen cells. Furthermore, progress in defining the properties and nature of the responsible immunizing factor is reported.

Another advance is revealed in three papers on cytotoxins. The problems of studies on these sera are clearly defined in one paper. One important question each investigator must face is whether the cytotoxins in which he is interested are true cytotoxic antibodies or other cytotoxic substances. Another paper describes the use of tissue cultures in such studies. It is a safe prediction that the technique of tissue culture, which has made outstanding recent contributions to medical science, will also prove useful in problems of immunity to cancer.

Some papers deal with immunity to virus-induced tumors. The virus problem in cancer research has received considerable attention in recent years, and immunity to these agents has a direct bearing upon the purpose of this monograph. One author warns that tests for neutralizing antibodies should be conducted with utmost care, because substances other than antibodies can neutralize viruses. This possibility must be kept in mind by those who attempt to reveal a virus in tumor tissues by means of this technique. Another investigator supports this warning by using a virus of the fowl leukosis complex to demonstrate that standard techniques of neutralization can be used to detect antibodies, provided the investigator is well acquainted with the physical, chemical, and biological properties of the virus with which he is working. Another paper describes practical procedures for quantitative studies with tumor viruses. These could and should be adapted to immunological studies.

Probably the best evidence of progress since 1929 revealed in this publication is the emphasis placed on problems of host-tumor relationships at the cellular level. The first three papers show this tendency. They expose the problems and present newer techniques for studying the role of cell components in the functions of cells and the importance of determining the relationships of the various components. All three papers describe advances that open opportunities for the study of cellular reactions to antigens and antibodies that were unheard of twenty-five years ago.

Four papers deal chiefly with the identification of tumor antigens. One emphasizes the specificity of the precipitin reaction, but it shows also that since the specificity of an antigen is determined by its chemical make-up, similar

qualitative reactions do not imply identical antigens because the antigens could contain the same or similar chemical groups. Theoretically this type of antigen could explain the nonspecificity of acquired immunity to propagable tumors.

Another paper describes a technique for analysis of antigen-antibody reactions in gels that enables the investigator to determine the number of soluble antigens present in tissue. This technique was used by another contributor who found some common antigens in various tissues and tumors, while others had a more limited distribution. These papers have suggested a new opportunity to approach the complexity of tumor antigens.

The fourth paper describes the use of fluorescent antibodies to detect the presence of antigens in cells. This technique may be useful in cancer research, especially if it can be used to test for antigens in spontaneous tumors.

Six contributors report work in which antibodies received most attention. One defines the use and limitations of complement fixation and reports progress in overcoming some of the difficulties. Two describe the use of radioactive substances to determine the localization of antibodies in tissues. Tumor-localizing antibodies are proving difficult because of the tendency of various foreign substances to localize in tumors, but a considerable degree of success has been achieved with one transplantable tumor.

Another contributor discusses the specificity of antibodies induced by extracts of thyroid glands. Those against normal thyroid tissue gave cross reactions with extracts of thyroid tumors. The relatively high degree of specificity of these antibodies suggests the possibility of detecting antigenic differences between normal and malignant tissues.

Two papers are concerned with the types of antibodies effective against tumor cells. One of them shows a strong predilection of such antibodies to localize in lymph nodes, and the other describes a technique whereby homografts have survived in diffusion chambers that denied entry to host cells, but have succumbed in chambers of larger pore size. Lymphocytes were implicated as the cell that entered the chambers in which grafts were destroyed. Heterografts, however, have not survived in cell-impenetrable chambers. Thus, humoral antibodies were probably involved in the destruction of the heterografts.

Immunity is one part of the problem of host-tumor relationships, and it has been an attractive one for many years, since it holds promise, as in other diseases, of preventive and diagnostic procedures. The contributors to this monograph have informed us of advances in the immunological approach to cancer control during the past twenty-five years, especially showing how the application of new and improved techniques has contributed to the problem. All of these techniques, however, have not been limited to the field of immunology. Almost all disciplines bearing upon cancer research have contributed to a better understanding of the cancer process and the complexities of host-tumor relationships. From this concentration of effort new techniques will be developed and new concepts will emerge to bring us closer to the control of the disease. Immunology will continue to play an important part for, in keeping with its contributions to the control of other diseases, it holds promise, not only of supplying preventive measures, but also of discovering clues to the etiology of cancer.

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